Organisation nucléaire et régulation transcriptionnelle dans les lymphomes

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Par

Diana MARKOZASHVILI

ORGANISATION NUCLEAIRE ET REGULATION TRANSCRIPTIONNELLE DANS LES LYMPHOMES

Thèse présentée et soutenue à Villejuif, le 9 décembre 2015

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I dedicate this manuscript to Achal Agrawal who gifted me with inner peace,

so crucial for the mind’s concentration.

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LIST OF ABBREVIATIONS

ADP — adenosine diphosphate

ALCL — anaplastic large-cell lymphoma

ALL — acute lymphoblastic leukemia

AMKL — acute megakaryoblastic leukemia

AML — acute myeloid leukemia

APL — acute promyelocytic leukemia

BL — Burkitt's lymphoma

CCND1 — cyclin D1 gene

ChIP — chromatin immunoprecipitation

CLL — chronic lymphocytic leukemia

CML — chronic myelogenous leukemia

CMML — chronic myelo-monocytic leukemia

CTCF — CCCTC-binding factor

CTCL — cutaneous T-cell lymphoma

DLCL — diffuse large-cell lymphoma

DNA — deoxyribonucleic acid

DNMT — DNA methyltransferase

DSB — double strand break

FACS — fluorescence-activated cell sorting

FAD — flavin adenine dinucleotide

FISH — fluorescence in situ hybridization

FL — follicular lymphoma

HAT — histone acetyltransferases

HDAC — histone deacetylase

HDACi — histone deacetylase inhibitor

HL — Hodgkin's lymphoma

IgH — immunoglobulin heavy chain

LCL — lymphoblastoid cell line

LSD — lysine-specific histone demethylase

MCL — mantle cell lymphoma

MDS — myelodysplastic syndrome

MLL — mixed-lineage leukemia

NAD — nicotinamide adenine dinucleotide

NBL — normal B-lymphocytes

NHL — non-Hodgkin's lymphoma

PRC — polycomb repressive complex

Pre-T-LBL — pre-T cell lymphoblastic leukemia/lymphoma

PTCL — peripheral T-cell lymphoma

qRT-PCR — quantitative real-time polymerase chain reaction

ROS — reactive oxygen species

SAM — S-adenosylmethionine

TF – transcription factor

T-PLL — pro-lymphocytic leukemia

TSA — trychostatin A

TSS — transcription start sites

U.S. FDA — United States Food and Drug Administration

VPA — valporoic acid

ABSTRACT

Mantle cell lymphoma (MCL) is a rare aggressive lymphoma caused by the chromosome translocation t(11;14)(q13;q32) juxtaposing the cyclin D1 (*CCND1*) locus on chromosome 11 with the immunoglobulin heavy chain (*IgH*) locus on chromosome 14. As a result, a proto-oncogene cyclin D1 which is not expressed in normal B-cells, becomes active. The initial hypothesis favored direct influence of the strong *IgH* enhancer on *CCND1* gene promoter to upregulate its transcription. However, the *CCND1* locus may be as far as 200 kb from the chromosome breakpoint. We have shown that 11q13 locus relocalizes from the nucleus periphery towards the transcriptionally active center and nucleolus (Allinne et al., 2014). This may lead to activation of the entire locus, suggesting an epigenetic mechanism of gene upregulation in MCL, rather than simple enhancer-promoter effect.

Several new treatments are proposed for MCL, including histone deacetylase inhibitors (HDACis) with epigenetic mechanism of action. In MCL cell lines, HDACis were shown to have antiproliferative effects and to decrease the cyclin D1 protein level in the cells. Until now, there is no clear understanding of this phenomenon, nor of HDACis mechanism of action. Therefore, a study of epigenetic state in 11q13 and 14q32 loci should significantly advance our knowledge about the mechanisms of cyclin D1 upregulation in MCL.

The purpose of the present work was to study chromatin structure in the rearranged (11;14)(q13;q32) locus in MCL cells as compared to the 11q13 and 14q32 loci in normal human B-lymphocytes. Furthermore, we studied the effect of different HDACis on the rearranged (11;14)(q13;q32) locus at several levels: histone modifications, chromatin conformation and gene expression.

We have shown that t(11;14)(q13;q32) translocation leads to overexpression of *CCND1* along with a group of genes spanning over 15 Mb around the translocation point. The genes sensitive to deregulation by t(11;14) translocation react to the HDACi treatment by increasing their expression. Importantly, while HDACi stimulates genome-wide disaggregation of heterochromatin, gene promoters stay shielded from its effect.

RÉSUMÉ

Le lymphome des cellules du manteau (LCM) est un lymphome d'une rare agressivité causée par la translocation chromosomique t(11;14)(q13;q32) juxtaposant le locus de la cycline D1 (*CCND1*) sur le chromosome 11 avec le locus de la chaîne lourde de l'immunoglobuline (IgH) sur le chromosome 14. En conséquence, une cycline D1 proto-oncogène devient active alors qu'elle n'est pas exprimée dans les cellules-B normales. L'hypothèse initiale semble indiquer une influence directe du fort enhancer IgH sur le promoteur du gène *CCND1* afin de surexprimer sa transcription. Quoi qu'il en soit, le locus *CCND1* peut être éloigné jusqu'à 200kb du point de cassure du chromosome. Nous avons montré que le locus 11q13 relocalise depuis la périphérie du noyau jusque au centre actif de transcription et au nucléole (Allinne et al., 2014). Ce phénomène qui mène à l'activation du locus entier, suggère un mécanisme epigénétique de régulation des gènes dans les LCM plutôt que simplement un simple effet enhancer-promoteur.

Plusieurs nouveaux traitements contre le LCM ont été proposés, y compris les inhibiteurs d'histone deacetylase (HDACis) qui impliquent des mécanismes epigénétique. Dans LMC, les HDACis sont décrites comme vaillant des effets antiprolifératifs et diminuant le niveau de la cycline D1 dans la cellule. Jusqu'à présent, les mécanismes d'action des HDACis reste obscurs. Pour ces raisons, une étude d'état epigénétique sur les loci 11q13 et 14q32 devrait fortement améliorer notre connaissance sur les mécanismes de surexpression de la cycline D1 dans les LMC.

L'objectif de ce travail est d'étudier la structure de la chromatine dans le locus réarrangé (11;14)(q13;q32) dans des cellules LMC par rapport au locus 11q13 et 14q32 dans les lymphocytes humains normaux. Nous avons ensuite étudié l'effet de différentes HDACis sur le locus réarrangé (11;14)(q13;q32) à plusieurs niveaux: l'acétylation / la méthylation des histones de la chromatine ainsi que sa conformation et l'expression des gènes.

Nous avons montré que t(11 ;14)(q13;q32) conduit à la surexpression de *CCND1* avec un groupe de gènes couvrant plus de 15 Mb autour du point de translocation. Les mêmes gènes, sensibles à la dérégulation par la translocation t(11;14), réagissent au traitement HDACi en augmentant leur expression. Nos résultats indiquent que bien que HDACi stimule la désagrégation de l'hétérochromatine sur l'ensemble du génome, les promoteurs de gènes restent à l'abri de ces effets.

РЕЗЮМЕ

Лимфома мантийной зоны (ЛМЗ) - редкая и агрессивная форма неходжкинской лимфомы, вызываемая транслокацией t(11;14)(q13;q32). В результате этой транслокации ген циклина D1 (*CCND1*) на 11 хромосоме соединяется с локусом генов тяжелых цепей иммуноглобулинов (*IgH*) на 14 хромосоме. Это вызывает активацию прото-онкогена циклина D1, что в норме не экспрессируется в В-лимфоцитах. Согласно изначальной гипотезе, непосредственное влияние сильного энхансера *IgH* вызывает гипер-экспрессию *CCND1*. Однако, *CCND1* может быть вынесен на расстояние до 200 kb от места разрыва хромосом. Ранее нами было показано, что локус 11q13 переносится во время транслокации с периферии ядра в транскрипционно активный центр рядом с ядрышком (Allinne et al., 2014). Это может повлечь за собой активацию всего локуса, указывая на эпигенетический механизм активации генов в ЛМЗ, нежели на простой эффект энхансера на промоутер.

В последние десятилетия были предложены новые методы лечения ЛМЗ, включая ингибиторы гистоновых деацетилаз (ИГД) с эпигенетическим механизмом действия. В клеточных линиях ЛМЗ было показано, что ИГД проявляют антипролиферативный эффект и парадоксально снижают количество циклина D1. До настоящего момента не существует объяснения этого явления, и точный механизм действия ИГД до конца не ясен. Поэтому изучение «эпигенетического ландшафта» в локусах 11q13 и 14q32 может существенно продвинуть наше понимание механизмов активации циклина D1 в ЛМЗ.

Целью данного исследования явилось изучение структуры хроматина в транслоцированном локусе (11;14)(q13;q32) в клетках ЛМЗ по сравнению с локусами 11q13 и 14q32 в нормальных В-лимфоцитах. Кроме того, мы исследовали эффект различных ИГД на измененный локус (11;14)(q13;q32) на нескольких уровнях: ацетилирование и метилирование гистонов, конформация хроматина и экспрессия генов.

В результате проведенных исследований мы показали, что транслокация (11;14)(q13;q32) приводит к гипер-экспрессии не только гена *CCND1*, но и большой группы генов, расположенных вокруг точки транслокации на 15 Mb фрагменте. Более того, эти же гены, что чувствительны к дерегулированию транслокацией t(11;14), реагируют на обработку ИГД повышением своей экспрессии. Мы показали, что несмотря на то, что ИГД вызывает глобальную диссоциацию гетерохроматина, промоутеры самих генов остаются незатронутыми, как бы защищенными от прямого влияния ингибитора.

Таким образом, полученные данные поддерживают гипотезу эпигенетического механизма, лежащего в основе дерегулирования экспрессии циклина D1 (что предоставляет рациональную базу для использования эпигенетических лекарств), и указывают на опосредованный механизм действия ИГД.

INTRODUCTION

I – The epigenetic machinery

Genetics studies heredity and variability of living organisms embedded in the sequence of DNA. In the last few decades however, other types of variations and inheritance which do not alter the underlying DNA sequence were found, called epigenetics. The term was derived from the Greek word epi- (επί-) which means 'over, outside of, around'. The definition of epigenetics was proposed by Conrad Waddington in 1942 (Waddington, 1942). Together with Ernst Hadorn (Hadorn, 1961), they were the first to assume an interaction between the environment and genetic information, which later evolved into the field now called epigenetics. This study shifts the focus of attention from a gene to the control over its activity.

Epigenetic mechanisms of gene expression include DNA CpG methylation, post-translational histone modifications, small and non-coding RNA-mediated regulation, chromatin remodeling and nucleosome repositioning. These epigenetic mechanisms work in deep interdependence and are orchestrated by the complex network of enzymes. Such modifications influence the overall chromatin structure, its activity and gene expression. The most broadly studied and well-characterized mechanisms currently are DNA methylation and histone modifications.

Recent studies established a link between epigenetics and complex diseases such as cancer, type II diabetes, schizophrenia, autoimmune disease and others. Indeed, changes in the pattern of DNA methylation or histone acetylation often precede or accompany malignant transformation (Esteller, 2011). New discoveries in this field open unexpected and promising

perspectives on the wellbeing of humans. International human epigenome projects are currently working to define the totality of epigenetic marks in all major tissues across the entire genome (Jones, 2008), (Zhang et al., 2011).

1. Chromatin Structure

To understand the epigenetic mechanisms, first the chromatin organization has to be appreciated (Figure 1). Genomic DNA in the eukaryotic cell is represented in the form of chromatin: DNA in the complex with associated proteins. 2 meters of DNA is wrapped around specialized histone proteins resulting in its packaging inside a cell nucleus with a diameter of ~10 μm. Such an organization makes DNA accessible to a variety of proteins involved in its transcription, replication and repair processes.

A stretch of double-stranded DNA 146 nucleotides long is wrapped around an octamer of the four core histones (H2A, H2B, H3, and H4) forming a nucleosome - a basic unit of chromatin. Amino-terminal tails of core histones extend from the globular centre of the octamer and are accessible to modifying enzymes (Luger et al., 1997). This form of chromatin compaction, known as "beads-on-a-string", is 10 nm wide. Similarly, nucleosomes fold into higher-order structures with the help of a linker histone H1 forming a 30 nm-diameter fiber, called a filament. There are two popular models of intermediate chromatin condensation: solenoid and zigzag models.

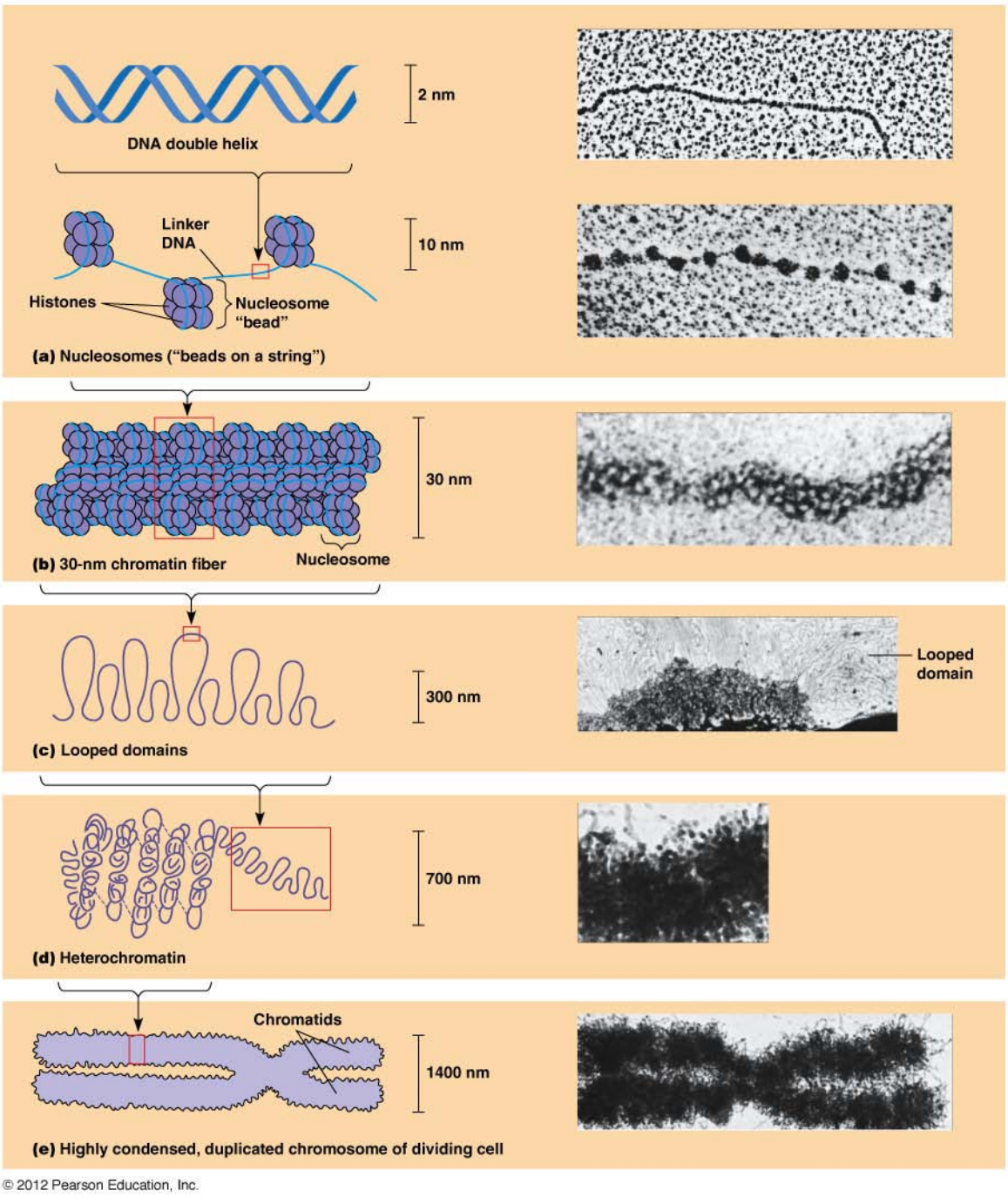


Figure 1. Chromatin compaction: from DNA to metaphase chromosome.

This scheme shows main levels of chromatin packing postulated to give rise to the highly condensed mitotic chromosome.

The 30 nm supercoiled chromatin forms independent loops by bounding anchor sequences to the nuclear matrix or scaffold. These anchor sequences, named S/MAR (scaffold/matrix attachment region), occur in 5´-introns and breakpoint cluster regions (BCRs). Unbound loop domains, approximately 300 nm in diameter, protrude away from the matrix or scaffold and play important role in gene expression and DNA replication processes (Heng et al., 2001). Domains of 300-700 nm, observed during interphase, together form a chromosome territory. Higher levels of chromatin organization are much less studied, and questions about how they are formed, regulated and interplayed with genomic activity remain elusive (Ruthenburg et al., 2007), (Razin et al., 2014).

There are several models proposed to describe the spatial arrangement of the chromatin within the nucleus. The modern hypothesis of the eukaryotic genome organization, first proposed in 1988 (Bodnar, 1988), (Goldman, 1988), is based on the conception of domains. According to this theory, genome is built up from units with similar structure and function (domains) which are controlled simultaneously mostly on the level of chromatin packaging (Razin et al., 2007). Importantly, 3D-organisation of genome defines spatial approaching of linearly-distant loci after folding into the higher order chromatin structures. Thus, pieces of one functional cluster can be discontinuous, but characterized by co-regulation and simultaneous switching between gene activation and repression, euchromatin or heterochromatin state.

The chromatin, from functional point of view, can be commonly divided into the more active euchromatin and the less active heterochromatin, where the former chiefly takes central position in the nucleus, whereas the latter segregates closer to periphery (**Figure 2**). Euchromatin represents a relaxed state of DNA which is more accessible to transcription factors and chromatin-associated proteins. It contains active and inactive genes, whereas heterochromatin mostly consists of inactive genes as it has a highly condensed inaccessible

form (Jenuwein and Allis, 2001). DNA sequences critical for chromosomal stability are as well represented in the heterochromatic state (Talbert and Henikoff, 2006).

Bivalent separation of chromatin into eu- and heterochromatin historically came from the experiments on sensitivity DNA to DNase I cleavage (Lawson et al., 1982), (Lawson et al., 1982). With the recent techniques of genome-wide analysis genome has been shown to be divided into more than two types of distinct active and inactive chromatin regions (Filion et al., 2010), (Farkash-Amar and Simon, 2010). Euchromatin is shown to be mostly composed of 30-nm fibers and looped domains, whereas heterochromatin represents more condensed chromatin domains or higher-order chromatin fibers, which are still not completely understood.

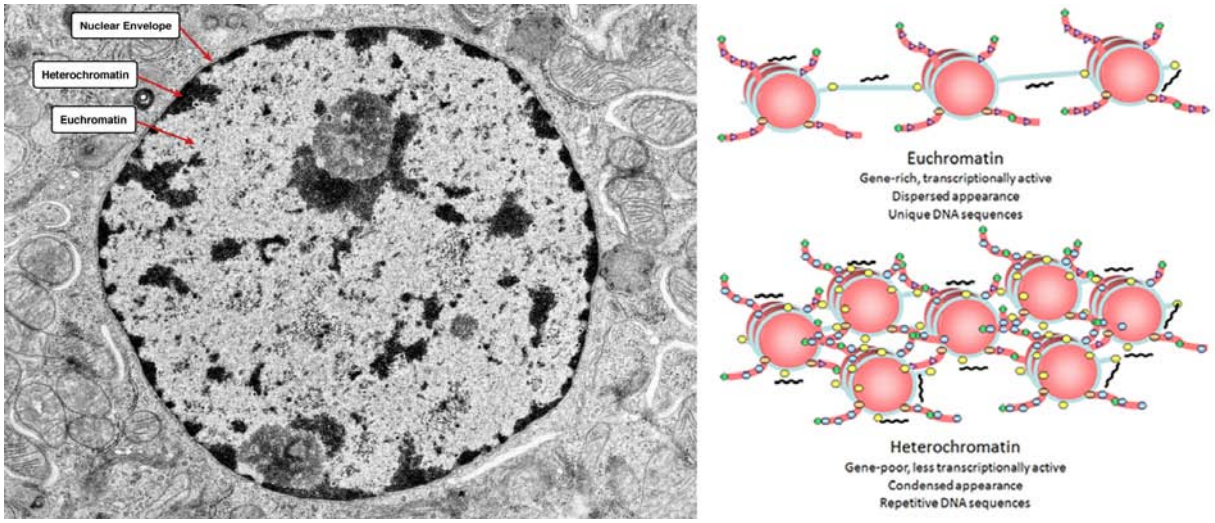


Figure 2. Euchromatin and heterochromatin.

Relative quantity of euchromatin and heterochromatin in a cell reflects the level of the cell's activity. Heterochromatin is accumulated adjacent to the nuclear envelope or scattered in irregular particles throughout the nucleus. Euchromatin is not readily stainable and dispersed in the center of nucleus.

2. Chromosome territories

All chromosomes have their own location in the nucleus according to their level of activity in different types of cells (Bolzer et al., 2005). These are called chromosomal territories (**Figure 3**). Initially, their existence had been suggested by Carl Rabl in 1885 (Rabl, 1885) and then proved in the early 1980s by Thomas and Christoph Cremer (Bodnar, 1988). The territorial organization of chromosomes in interphase turns out to be a basic feature of nuclear architecture, specific to both cell and tissue type, and are evolutionary conserved (Tanabe et al., 2002).

Chromosome territories are radially arranged around the nucleus and were shown to correlate with their gene density and size. In this case, more active and gene-rich territories segregate in the centre, whereas more silent with less genes density territories – close to the periphery of the nucleus (Sun et al., 2000). Notably, once a gene is “switched on” it can be relocated from the periphery towards the interior (Chuang et al., 2006). The same is also true for the opposite process. This phenomenon shows the dynamical structure of chromosome territories.

The chromosomal territories are comprised of higher order chromatin domains called Topologically Associating Domains (TADs) of ~1 Mb each (Albiez et al., 2006). These 1Mb domains are likely to be built up from smaller loop units, and at the same time, can themselves serve as smaller units for larger chromatin clumps (Cremer and Cremer, 2010).

If part of a chromosome relocalizes into the new atypical surroundings, as it happens in case of translocations (Harewood et al., 2010), (Allinne et al., 2014), then this change of usual position causes an aberrant gene expression in most of the cases. Such repositioning is often observed

in different diseases. For example, the majority of lymphomas contain a chromosomal translocation with consequent overexpression of a proto-oncogene.

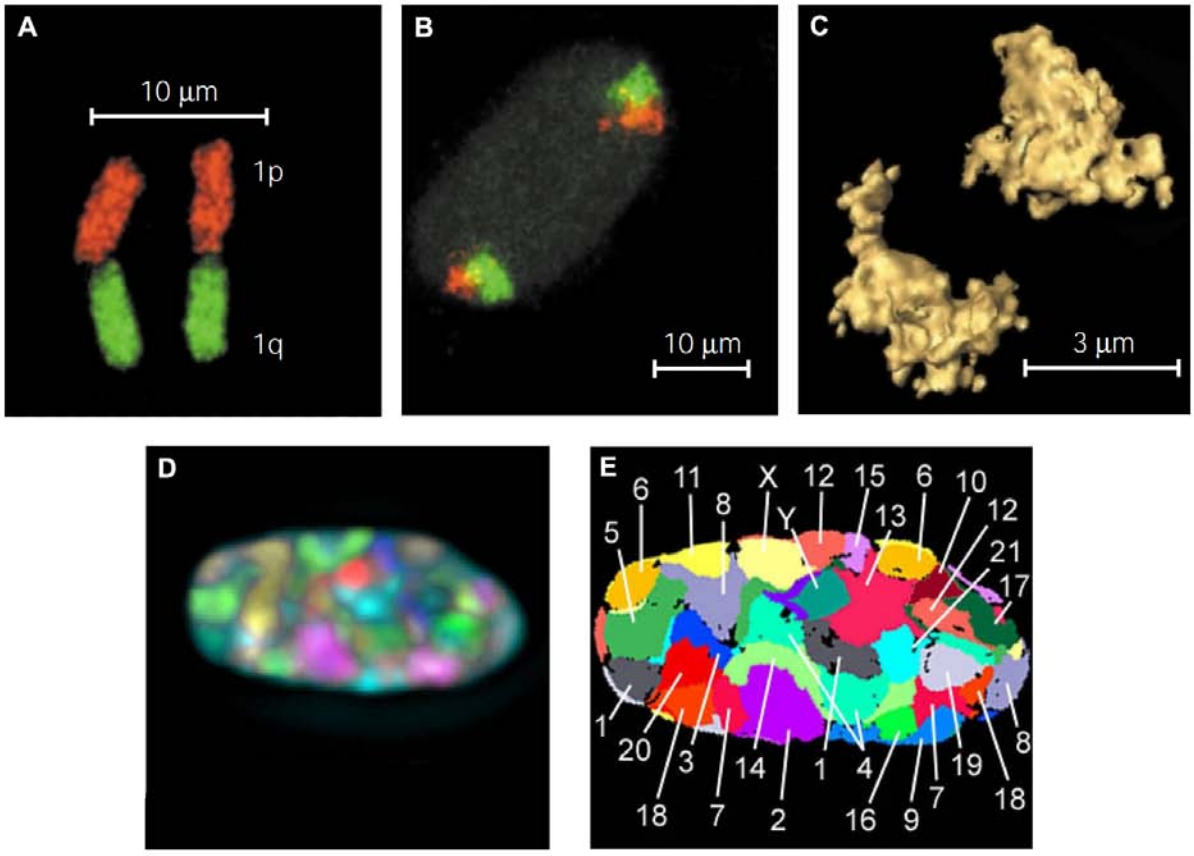


Figure 3. Chromosome territories.

(A) Two-color painting of the p-arm (red) and the q-arm (green) of human chromosome 1 in a lymphocyte metaphase spread. (B) Visualization of the two arms in a light optical section through a human diploid fibroblast nucleus (bottom) shows two distinct, mutually exclusive arm domains. (C) Three-dimensional reconstructions of two chromosome 17 territories, established from light optical serial sections through a human diploid fibroblast nucleus, show complex territory surfaces. (D-E) 24 colors FISH representation of all visible chromosome territories in a nucleus. [from (Cremer and Cremer, 2001) and (Bolzer et al., 2005)]

3. Levels of epigenetic regulation

The dynamic composition of chromatin during different stages of the cell cycle, or from one cell type to another, is regulated through multiple epigenetic mechanisms.

a. DNA methylation

In higher eukaryotes, the cytosine within the dinucleotide CpG can be methylated. Perhaps it is the best characterized chemical modification of chromatin. DNA methylation plays a critical role in gene imprinting, tissue-specific gene expression, genome stability, cell differentiation, X chromosome inactivation, regulation of chromatin structure, carcinogenesis, and aging (Bird, 2002). The process involves the transfer of a methyl group from S-adenosyl-L-methionine (SAM), a methyl precursor, to the cytosines in CpG dinucleotides. The latter are often clustered in the genome in the so-called CpG islands ranging from 0.5 to 5 kb in size. These sites tend to cluster in regions of large repetitive sequences such as centromeric repeats or at the 5' ends of many genes (Bernstein et al., 2007).

Enzymes responsible for DNA-methylation are called DNA methyltransferases (DNMTs). The human genome contains four DNMT genes, DNMT1, DNMT2, DNMT3a and DNMT3b (**Figure 4**). Two formers DNMT are *de novo* methyltransferases, preferentially targeting unmethylated CpGs to initiate methylation (Stresemann et al., 2006). Once established in early embryogenesis, DNA methylation patterns must be stably maintained over cell divisions. This function is fulfilled by DNMT1, the maintenance methyltransferase, through its preference for hemimethylated DNA. DNA methyltransferase DNMT2 exhibits a weak methyltransferase

activity in vitro and has only one catalytic domain, whereas DNMT1 and DNMT3A/B enzymes contain both regulatory and catalytic domains (Li and Zhang, 2014).

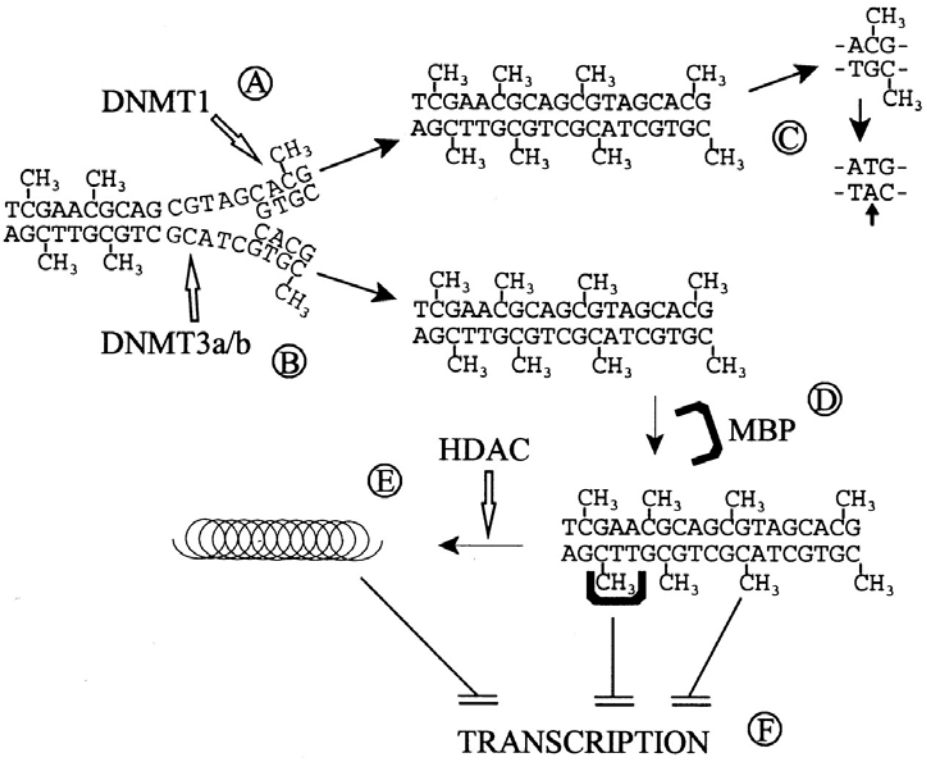


Figure 4. DNA methyltransferases.

(**A**) DNA methyltransferase 1 (DNMT1) predominantly methylates hemi-methylated DNA during replication. (**B**) DNMT3a and DNMT3b predominantly methylate unmethylated DNA. (**C**) 5-Methylcytosine can undergo spontaneous deamination to thymine, generating T:A transition mutations. (**D**) Methyl-CpG binding proteins (MBPs) bind to methylated DNA; (**E**) DNMT1 and MBPs can recruit histone deacetylase, resulting in tighter packing of DNA into chromatin. (**F**) Methylation, MBPs and histone deacetylation inhibit transcription by interfering with transcription factor access. [from (Goffin and Eisenhauer, 2002)]

The opposite process, demethylation of DNA, can be passive or active. Passive DNA demethylation happens when maintenance methyltransferases are inactive and newly synthesized strand, after DNA replication, retains the unmethylated state. Active DNA

demethylation can occur independently of DNA replication and involves several enzymes, which have been elusive for many years. Methyl-CpG-binding domain protein 2 (MBD2), one of the first suggested enzymes responsible for DNA demethylation, is now disputed. Recently, a mechanism of 5meC excision in plants involving DNA glycosylases was discovered, although evidence supporting a similar mechanism in mammals has been less forthcoming. DNA demethylation can also be achieved by deamination of 5meC with the help of Aid and APOBEC enzymes generating thymine (T). This is followed by consequent replacement of mismatched T with unmethylated C. Alternatively, TET enzymes can oxidize 5mC with production of 5-hydroxymethyl cytosine (5hmC) (**Figure 5**). Modified bases in both processes of deamination and oxidation are removed by glycosylases with generation of apyrimidinic acid which is subsequently replaced with cytosine (for review see (Wu and Zhang, 2010)).

Interestingly, 5-hydroxymethyl cytosine functions not merely as a passive intermediate in the DNA demethylation pathway. 5hmC serves as an epigenetic mark opposing DNA methylation and plays important role in stem cell renewal, neurological disorders, cancer development and progression (Rajneesh and Sinha, 2014). For these reasons, 5hmC is sometimes referred to as the “sixth base”.

Methylation of DNA has many roles in various cellular processes and may impact the transcription of genes by preventing the binding of key transcriptional factors. Indeed, control elements for transcription and replication often contain CpG islands (Bird, 2002), (Prioleau, 2009), which recruit methyl CpG-binding transcriptional repressors and interfere with DNA binding of transcriptional activators. This results in a condensed chromatin state. Normally in heterochromatic regions, 50-70% of all CpGs are methylated, whereas in euchromatin within promoters, they typically remain unmethylated, especially during development and in normal (non-neoplastic) tissues.

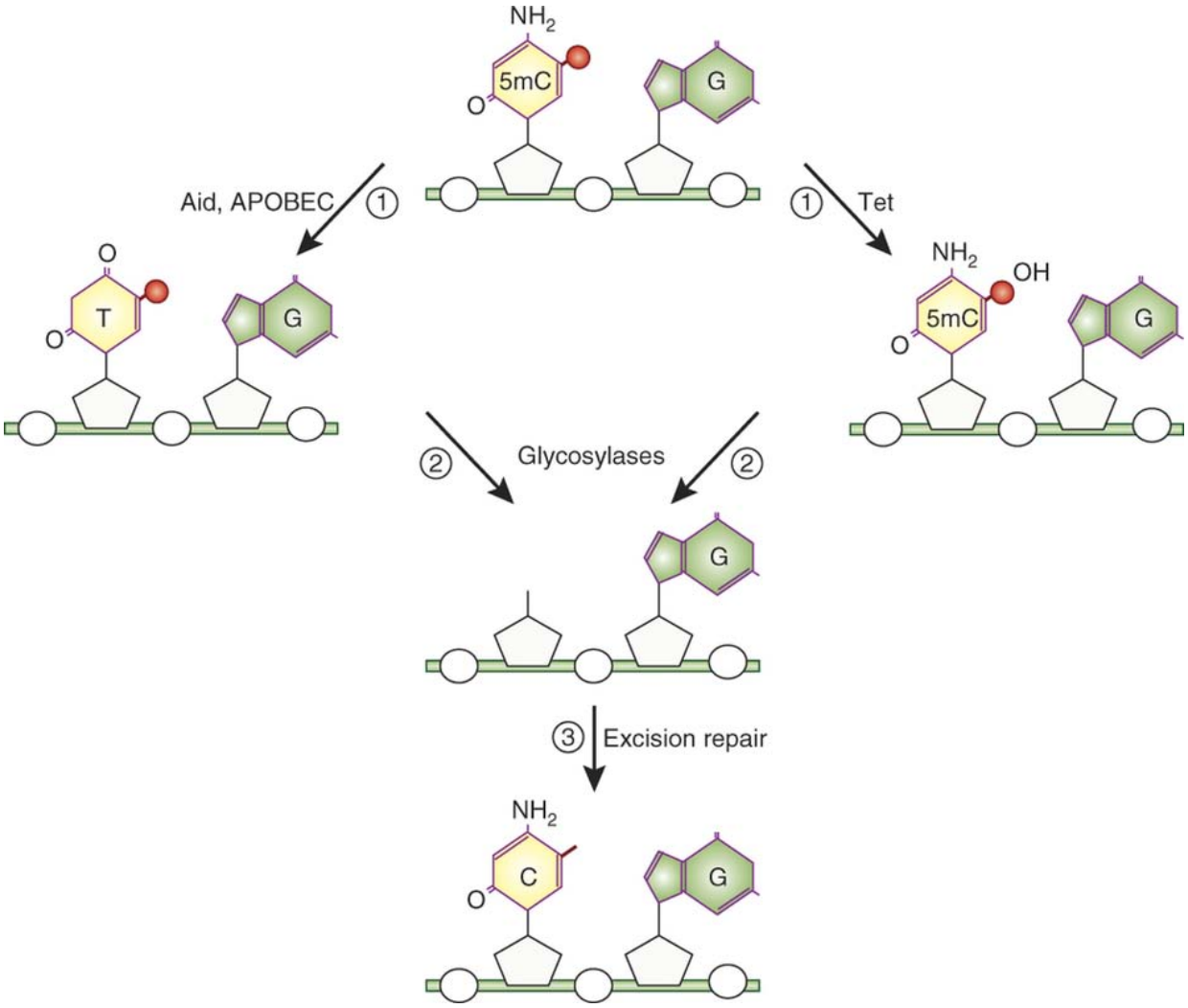


Figure 5. Oxidation and deamination pathways of demethylation.

Supposed mechanisms of demethylation via modification of 5mC through deimination (Aid, APOBEC) or hydroxymethylation (Tet). These modified bases are removed by glycosylases, which generate apyrimidinic acid that is subsequently removed by excision repair and replaced with cytosine. [from (Bergman and Cedar, 2013)]

DNA methylation patterns are dynamic and change intensively during development. In primordial germ cells, genome is actively demethylated. After fertilization in the early embryo, methylation levels increase rapidly and establish a differential pattern in the trophectoderm and

the inner cell mass (Jaenisch et al., 1982). This type of *de novo* DNA-methylation was as well observed in adult somatic cells during aging, or development of disease like cancer (Issa, 2000). Apart from that, CpG-island methylation can be developmentally programmed, like in cases of X-chromosome inactivation, genomic imprinting and tissue-specific differentiation (Laird, 2003).

After being established in early development, DNA methylation pattern tends to be copied during the DNA replication process in a semiconservative way, thus passing the epigenetic information between cell generations (Holliday and Pugh, 1975). Recent discoveries revealed that this process though might be not so simple. Apparently, DNA methyltransferases have high frequency of mistakes (~4% per cell division). In spite of the high error rate at the single CpG methylation level, the general methylation pattern is propagated during development at a significant rate (Bird, 2002). Moreover, it has been shown that CpG-island methylation can still be maintained without the DNMT1 which is known to be responsible for the process (Rhee et al., 2000). This hints on a yet unknown component critical for the maintenance process.

Several lines of evidence suggest that DNA methylation affects genes which are already silent (Gautsch and Wilson, 1983), (Pannell et al., 2000). For example, phosphoglycerate kinase gene in mammals is silenced on inactive X chromosome before methylation of its promoter occurs (Lock et al., 1987). When DNA methyltransferase 1 (an enzyme responsible for maintenance of methylation) is inhibited, X-linked transgene undergoes the frequent reactivation in mouse embryo cells and in cultured somatic cells (Sado et al., 2000). These and many other data indicate that methylation serves rather as a secondary event which aims to silence DNA irrevocably.

Even though DNA methylation has been repeatedly shown to interfere with transcription in a mutually exclusive way (Bird, 2002), emerging evidence for existence of the opposite processes recently appeared (Rothbart and Strahl, 2014). Transcription factors binding specific DNA sequences in a methylation-dependent manner have been identified. For example, recognition of 5mC by KLF4 plays a stimulatory role in KLF4-mediated transcription. New intriguing findings expand the functional role of 5mC in gene regulation: it might mediate both active and repressive gene states but in a site-specific manner.

b. Non-coding RNA

In mammals only 1% of genome codes for proteins (Taft et al., 2007), whereas most part of DNA is still being transcribed without consequent translation. That 'transcriptional noise' or 'junk DNA' has been found to be evolutionarily conserved and to serve for regulation of gene expression and genome stability (Guttman et al., 2009), (Costa, 2007). Indeed, 98% of all transcriptional output results in so called non-coding RNA (ncRNA) (Mattick, 2001), which is synthesized by RNA polymerase II and III (Dieci et al., 2007).

As research progresses in this field, more and more different types of ncRNAs have been identified (Zhou et al., 2010). 2 main groups of non-coding RNAs include the short ncRNAs (<30 nts) and the long ncRNAs (>200 nts). The major classes of short non-coding RNAs include: microRNAs (miRNAs), short interfering RNAs (siRNAs), and piwi-interacting RNAs (piRNAs). Many of ncRNAs are involved in the processing and regulation of other RNAs such as mRNA, tRNA, and rRNA, and chromatin. A mechanism by which RNA molecules specifically inhibit gene expression is called RNA interference (RNAi). In addition to this

function, ncRNAs play an important role in defending cells against parasitic nucleotide sequences – viruses and transposons.

miRNAs are tiny and highly conserved ncRNAs that induce degradation of specific mRNAs or repression of translation by complementary binding to a target. Being produced from either their own genes or from introns as single-stranded RNAs, they are then processed by the RNase III endoribonuclease (Dicer). Together with RNA-induced silencing complex (RISC), these RNAs bind to a specific target mRNA inducing its cleavage, degradation or blocking its translation. miRNAs control approximately 30% of all human protein-coding genes from almost every cellular process (Filipowicz et al., 2008). In addition, miRNAs were reported to regulate expression of other types of ncRNAs, such as long ncRNAs (Calin et al., 2007).

siRNAs, similarly to miRNAs, mediate transcriptional and post-transcriptional gene silencing and are involved in the process of chromatin modification (Carthew and Sontheimer, 2009). These molecules are as well catalysed by the Dicer enzyme, but initially synthesized as dsRNAs with a process of bi-directional transcription (Katayama et al., 2005).

piRNAs originate form the largest class of small ncRNAs and regulate the suppression of transposon activity in germline and somatic cells. piRNAs in complexes with piwi proteins target transposons in antisense way and cause its cleavage (O'Donnell and Boeke, 2007). These RNAs are produced as single strand clusters and then cleaved to individual units through a yet undefined processing mechanism (Houwing et al., 2007).

Long ncRNAs (lncRNA) have a length of more than 200 nucleotides and represent the major group of non-coding RNAs. The most common function of the lncRNAs, together with chromatin-modifying proteins, is to create a repressive chromatin state and influence gene expression (Mercer and Mattick, 2013). The most known example is X-chromosome

inactivation which involves two lncRNAs: Xist and its negative regulator - antisense transcript Tsix (Lee, 2009). Coating of X-chromosome with Xist transcripts triggers extensive histone methylation and chromosome inactivation. Notably, implication of lncRNAs in gene activation by establishing transcriptionally competent chromatin structure as well has been reported (Guttman et al., 2011).

Non-coding RNAs play a significant role in epigenetic regulation of gene expression via mechanisms such as RNA-associated gene silencing, DNA methylation, chromatin remodelling, chromosome inactivation, genomic imprinting and paramutation (**Figure 6**) (Zhou et al., 2010).

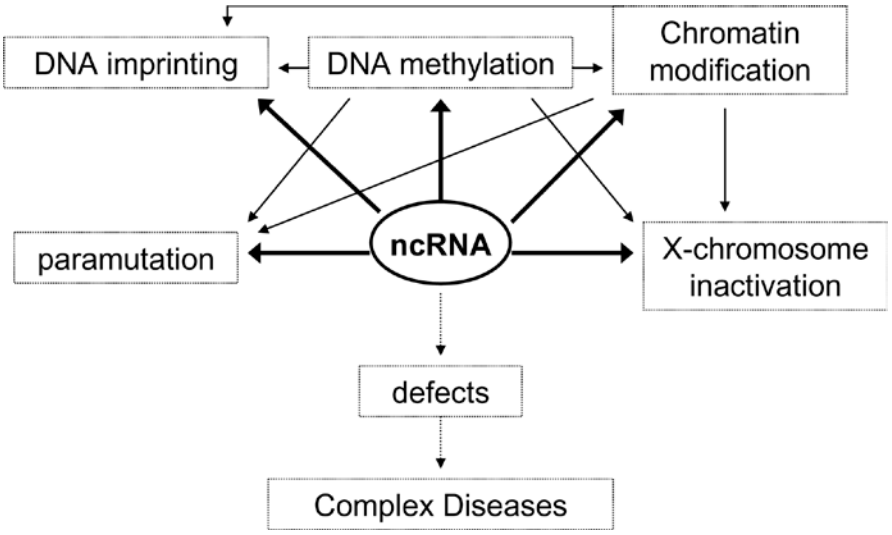


Figure 6. Schematic representation of the different components of epigenetic control by ncRNAs in epigenetic regulation. [from (Zhou et al., 2010)]

c. Histone modifications

The amino terminals of the core histones can be subjected to several types of modifications, including acetylation of lysines (K), methylation of arginine (R) and lysines, phosphorylation of serine (S) and threonine (T), ubiquitination and sumoylation of lysines, etc. (Kouzarides, 2007). These post-translational modifications of histones are critical for global chromatin environments and the orchestration of DNA-based biological tasks. Regulation of chromatin structure and function affects chromosomal organization, gene transcription, recombination, DNA repair and replication.

All these biological events require ordered recruitment of the machinery to unravel DNA, manipulate it and then put it back to the correct chromatin state. These processes have different steps, wherein each of them requires a distinct type of chromatin-remodeling activity. This explains the necessity of different combinations of modifications. To describe the specific set of histone modifications required for a given DNA function, the term “histone code” has been proposed (Jenuwein and Allis, 2001).

The abundance of modifications on the histone tails creates a ‘‘crosstalk’’ between them (**Figure 7**). First, if more than one possible modification targets the same site, it may lead to competitive antagonism, as in the case of lysines which can be methylated, acetylated or ubiquitylated. Second, one modification can be dependent on another, being stimulated or disrupted by a specific proteins binding to modified residues.

Histone modifications function via two different mechanisms. The first is regulation of chromatin folding intensity. The second is recruitment of nonhistone proteins which specifically bind the modified histones. Thus, acetylation is recognized by bromodomains and nonrelated PHD

domains, methylation - by chromo-like domains, and phosphorylation is recognized by a domain within 14-3-3 proteins (**Figure 8**) (Kouzarides, 2007).

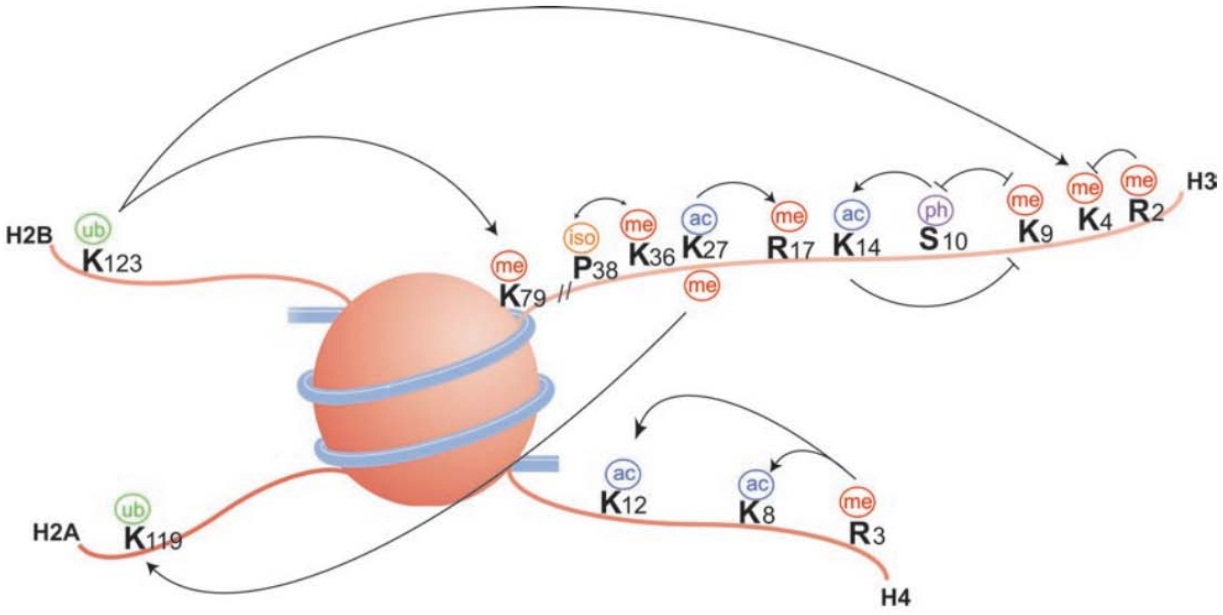


Figure 7. Histone modification cross-talk.

Histone modifications can positively or negatively affect other modifications. A positive effect is indicated by an arrowhead and a negative effect is indicated by a flat head [from (Bannister and Kouzarides, 2011)]

- ***Histone methylation***

Histone methylation occurs on different lysine residues. Depending on the number of the methyl groups (from one to three) and on the lysine residues' positions, it exerts a different effect on gene function and chromatin state. Histone methylation can be associated both with transcriptional activation and inactivation, as well as with silent genomic regions (Jenuwein and Allis, 2001).

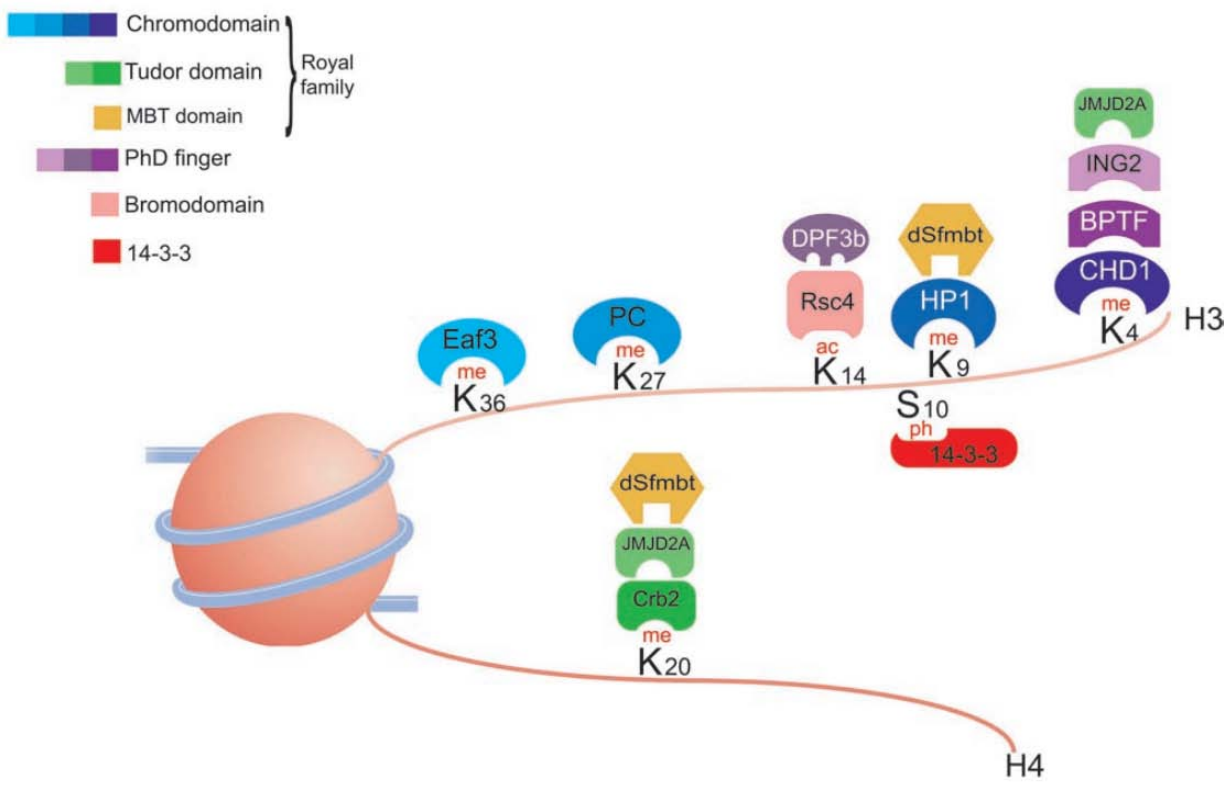


Figure 8. Domains binding modified histones.

Examples of proteins with domains that specifically bind to modified histones as shown. [from (Bannister and Kouzarides, 2011)]

For example, modifications that are localized to active genes, such as H3K4me, H3K36me, or H3K79me, are often referred to as euchromatin modifications (Koch et al., 2007), (Edmunds et al., 2008). In contrast, the enrichment of histone methylation at H3K9me, H3K20me, or H4K27me is associated with gene inactivation or silencing (Kouzarides, 2007). Co-existence of H3K4me3 and H3K27me3 marks keeps genes in poised states in so called "bivalent domains" for later activation in stem cells (Meissner et al., 2008), (Mikkelsen et al., 2007). Interestingly, H3K9me1, H3K27me1 and K4K20me1 are documented to be implicated in gene activation and were observed in euchromatin regions, whereas with increased methylation degree these

modifications H3K9me3, H3K27me3, and K4K20me3 are associated with transcriptional repression and heterochromatin formation (Barski et al., 2007), (Vakoc et al., 2006).

Apart from the transcription regulation, several lines of evidence suggest that lysine methylation of histone proteins also functions to maintain genome integrity and cellular identity (Albert and Helin, 2010). For example, H3K9 methylation plays crucial role in the maintenance of chromosome integrity (Peters et al., 2001).

- ***Histone acetylation***

The acetylation of histones is the most widely studied histone modification and is the first epigenetic modification connected with biological activity (Feinberg and Tycko, 2004). Lysine residues have a positive charge that can bind tightly to the negatively charged DNA. That process is responsible for formation of condensed nucleosomes and closed chromatin structure inaccessible to transcriptional machines. Acetylation has the effect of changing the basic lysine charge from positive to neutral, thus transforming DNA to a more accessible state (Shahbazian and Grunstein, 2007). All four core histones are susceptible for that modification.

Hyperacetylation is involved as well in recruitment of other chromatin associated factors which participate in gene transcription or chromatin architecture (Ruthenburg et al., 2007). In contrast, histone deacetylation leads to opposite effects. Thus, acetylation of histones is known to be exclusively associated with active chromatin.

Moreover, recent studies have revealed a link between histone acetylation and nucleosome structure and dynamics (Tessarz and Kouzarides, 2014). Acetylation of H3K122 or H3K64 correlates with decreased nucleosome stability.

- ***Other histone modifications***

Phosphorylation of serine residues, like in case of acetylation, changes its basic charge and thus may have important consequences for chromatin compaction and cellular processes such as mitosis, apoptosis, and gametogenesis (Fischle et al., 2005), (Krishnamoorthy et al., 2006), (Ahn et al., 2005). During cell division, histones are often phosphorylated at specific sites (Barber et al., 2004). Phosphorylation of H2A in mammalian cells takes place as an early response to DNA damage (Fillingham et al., 2006). Phosphorylation of threonine can markedly influence nucleosome architecture. For example, H3T118 phosphorylation can induce the formation of alternative nucleosome arrangements (North et al., 2014).

Ubiquitylation. Lysine 119 in H2A, lysine 120 in H2B and histone H3 (the exact site has not yet been indentified) in humans are susceptible to ubiquitylation. Modification of H2AK119, mediated by the Bmi/Ring1A protein, has been found to be associated with transcriptional repression (Wang et al., 2006). In contrast, H2BK120 ubiquitylation was linked with transcription activation and elongation (Zhu et al., 2005), (Pavri et al., 2006). H2B monoubiquitination is required for proceeding of histone methylation, a process known as histone crosstalk. Apart of that, ubiquitylation was shown to play a role in UV-induced DNA repair (Wang et al., 2006).

Sumoylation is a very large modification which can take place on all four core histones (Nathan et al., 2006). It was shown to be antagonistic for both acetylation and ubiquitination, as it occurs on the same lysine residue. Consequently, this modification is repressive for the transcription.

ADP-Ribosylation is a reversible addition of one or more ADP-ribose moiety to an arginine residue in histones. This modification is involved in many cellular processes, including cell

signaling, DNA repair, gene regulation and apoptosis (Hassa et al., 2006). Improper ADP-ribosylation has been implicated in some forms of cancer (Scarpa et al., 2013).

Citrullination has recently been reported to be implicated in histone–DNA interactions (Christophorou et al., 2014). Citrulline is derived from arginine amino acid by peptidylarginine deimination with loss of its positive charge. In embryonic cells linker histone H1 is citrullinated at the position Arg54 in a domain necessary for the interaction with nucleosomal DNA. This is apparently one of the reasons for more accessible chromatin in embryo cells as compared to differentiated cells.

4. Enzymes involved in histone modifications

Covalent modifications of histones, ranging from small chemical changes such as methylation and acetylation to large peptide addition such as ubiquitylation and sumoylation, add multiple layers of complexity to chromatin. Most part of histone modifications are dynamically regulated, which was demonstrated by the discovery of many enzymes that can remove the modification. Over the past decade, several families of histone-modifying enzymes have been identified.

a. Histone methyltransferases / demethylases

Histone methylation ensures binding of regulatory proteins that affect chromatin structure in different ways. Lysine-(K) and arginine-(R) residues in histone proteins can be methylated (Martin and Zhang, 2005), (Bedford and Clarke, 2009). Amongst all the enzymes that modify

histones, those responsible for methylation / demethylation are the most specific about their histone targets. Reversible methylation is catalyzed by S-adenosylmethionine (SAM) - dependent methyltransferases and erased by two classes of demethylases: either hydroxylases of Jumonji C family or amine oxidases - the lysine-specific demethylases 1 (LSD1) and 2 (LSD2) (Tsukada et al., 2006); (Shi et al., 2004b).

Histone methyltransferases (HMT) are divided based on their substrate, into lysine or arginine-specific types. Both types belong to the family of enzymes with a conserved catalytic domain called SET (Suppressor of variegation, Enhancer of Zeste, Trithorax), except histone lysine methyltransferase DOT1L, which does not contain a SET domain. All histone methyltransferases use SAM as a cofactor and methyl donor group.

In human genome, there are approximately 50 HMTs, and each enzyme regulates different genes or different cellular processes. For instance, methyltransferases Set1 and Set2 methylate Lys4 and Lys36 of histone H3 respectively (Shilatifard, 2006). Dot1, a SET-domain free HTM, catalyzes histone H3K79 mono-, di- and trimethylation, which mediates telomere silencing (Lacoste et al., 2002). Dot1, unlike SET-containing HMTs, can methylate a lysine residue in the globular core of the histones, whereas others do so only in the tail region of the histone (Wood and Shilatifard, 2004).

The first histone demethylase (HDM) discovered was Lysine Specific Demethylase 1 (LSD1) specific for mono- and dimethylated Lys4 in histone H3 (Shi et al., 2004a). LSD1 and LSD2 belong to the conservative flavin adenine dinucleotide (FAD)-dependent amino oxidase family, which are specific for mono- and dimethyllysines only.

Next discovered HDMs were reported to contain a JmjC catalytic domain, known to be implicated in chromatin-dependent functions (Clissold and Ponting, 2001). In contrast to

LSD1/2 demethylases, these JmjC enzymes are able to catalyze trimethyllysines as well as mono- and dimethylated substrates (Klose and Zhang, 2007). Many JmjC family members have unique substrate specificities.

Histone arginine demethylases could not be identified for a long time. Recently, a human JMJD6 was shown to possess an arginine-specific demethylated activity specific for H3R2me1/2 and H4R3me1/2 (Chang et al., 2007).

b. Histone acetyltransferases

Histone acetylation is a reversible process catalyzed by histone acetyltransferases (HAT) and histone deacetylases (HDAC). HATs use acetyl-CoA as a cofactor to acetylate ε-amine of either arginine-(R) or lysine-(K) residues. In a cell, histone acetyltransferases can be localized in the nucleus, acetylating histones in chromatin (HAT type A), and in the cytoplasm, facilitating assembly of nucleosomes by acetylating newly translated histones (HAT type B) (Richman et al., 1988).

Acetylated chromatin has a relaxed structure which is favorable for transcription and may interact with bromodomain-containing family of proteins that, in turn, can act as transcription activators or chromatin remodelers (Filippakopoulos et al., 2012). Moreover, many transcriptional coactivators, such as CBP/p300, Gcn5/PCAF and SRC-1, have been shown to possess intrinsic HAT activity.

In total, there are about 30 known human HATs which can be divided into 5 families based on structural and functional homology of their catalytic domains. GNATs, Gcn5-related N-

acetyltransferases, have 4 conserved motifs in their HAT domain and bromodomain or chromodomain for binding acetylated or methylated lysine, respectively (Neuwald and Landsman, 1997). GNATs are usually involved in cellular growth (Zhang et al., 1998). MYST (MOZ, Ybf2, Sas2, and Tip60) HATs contain acetyl-CoA binding motif and a zinc finger in their MYST- and other domains for interaction with other proteins (Avvakumov and Cote, 2007). In general, MYST HATs are involved in survival, transcription and cell growth control. This HAT family is closely linked to cancer (for a review see (Avvakumov and Cote, 2007)). The other three families include: the p300/CBP HATs; the steroid receptor co-activators (SRC)/nuclear receptor co-activators (NCoA) family; and the general transcription factor HATs containing TAF250 domain (Torchia et al., 1998).

HATs are often not specific to individual lysines, but nevertheless fulfill specific functions. For instance, both the elongator complex and SAGA complex acetylate H3K9 and H3K14, where former functions in coding regions, while latter acts at promoters (Wittschieben et al., 1999). It can be explained by the fact that HATs are often revealed to be a part of multisubunit protein complexes that determine their recruitment, catalytic activity and substrate specificity (Nagy and Tora, 2007).

HATs carry out a broad range of functions acetylating not only histones, but also an increasing number of non-histone substrates (Yang, 2004). For example, tumor suppressor p53 is regulated by acetylation carried out with p300/CBP HAT (Gu and Roeder, 1997).

c. Histone deacetylases

The removal of acetyl groups from arginine and lysine residues in histones is catalyzed by histone deacetylases (HDACs). This group, comprising of 18 humans enzymes, is quite heterogeneous and can be divided into four classes based on their sequence homology to their yeast orthologues: Rpd3, yHda1 and Sir2 (**Table 1**).

The Class I, II, and IV HDACs, called as well «classical» HDACs, belong to the arginase/deacetylase superfamily of proteins which require Zn2+ for their catalytic activity (**Figure 9)**. Class I HDACs are localized in the nucleus and are the most abundant and ubiquitously-expressed. Class II HDACs shuttle between the nucleus and the cytoplasm and their expression is tissue-specific. That class can be divided into two sub-classes: IIa which is characterized as having a highly conserved C-terminal deacetylase catalytic domain and a unique N-terminal domain; IIb which contains two deacetylase domains. Class IV has only one member HDAC11 and resides in the nucleus (Kim and Bae, 2011). Class III, or sirtuins, belongs to deoxyhypusine synthase like NAD/FAD-binding domain superfamily and uses NAD+ as an essential co-factor. Apart from histone deacetylation, sirtuins have mono-ADP-ribosyltransferase enzymatic activity (Seto and Yoshida, 2014)

When purified to homogeneity, classical HDACs reveal very low histone deacetylase activity which makes it difficult to define their histone substrate specificity. HDACs exist as a part of several different complexes, where each of them shows different substrate preferences. In contrast to the classical ones, class III HDACs have clearer histone substrate specificity (Table 1).

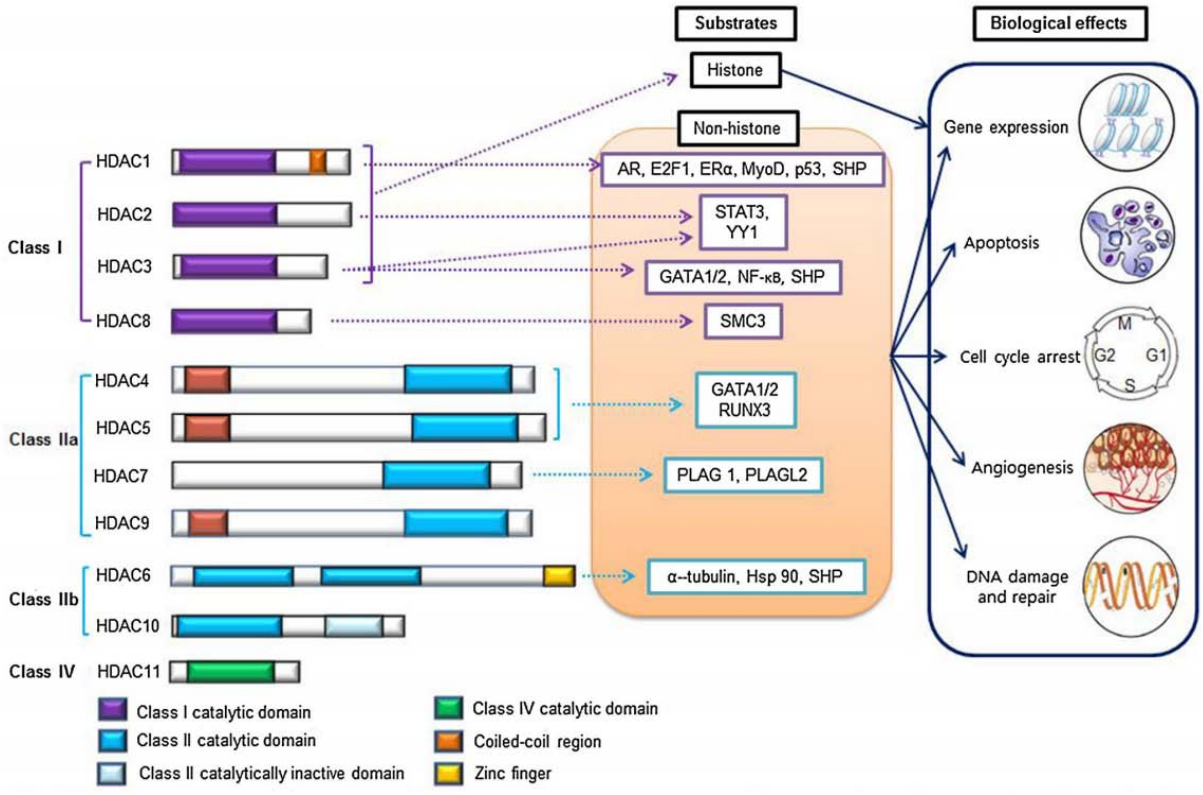


Figure 9. The molecular targets of HDACs and the biological effects of HDAC inhibition.

HDAC inhibitors induce acetylation of histones and non-histone proteins, leading to a wide range of biological effects, including regulation of gene expression, including apoptosis and cell cycle arrest, inhibiting angiogenesis, and regulation of DNA damage and repair pathway. AR, androgen receptor; ERa, estrogen receptora; NF-KB, nuclear factor-KB; PLAG1, pleomorphic adenoma gene 1; PLAGL2, PLAG-like 2; RUNX3, runt-related transcription factor 3; SHP, short heterodimer partner; SMC3, structural maintenance of chromosomes 3; STAT3, signal transducer and activator of transcription 3; YY1, Ying Yang 1 [from (Chun, 2015)]

All types of HDACs, apart from regulating histone modification, also regulate a wide range of non-histone proteins working in nucleus, cytoplasm and mitochondria: such as chaperones, transcription factors and structural proteins (Witt et al., 2009a); (Seto and Yoshida, 2014). For example, acetylation of Lys residue competes with its other modifications, thus inhibiting ubiquitination dependent protein degradation (Caron et al., 2005). Recently, a large number of

Table 1. Human HDACs and their functions.

HDAC	Protein associations (normal and oncogenic)	Role in the cell	Expression in tumor tissues	Function in cancer cells	Links
Class I (homologous to RDP3 yeast protein, nuclear location, ubiquitous tissue expression, co-factor for activity - Zn2+)					
HDAC1 (482 a.a.)	HDAC2, CoREST, NuRD, Sin3, AML1-ETO, PML, PLZF, BCL6, p53, AR, ER, Rb/E2F1, MyoD, STAT3, androgen	Represses transcription, binds to TF. Resistance to chemotherapy Proliferation control Apoptosis p21 and p27 CDK inhibitor repression	Especially common in cancers of the gastrointestinal system and is associated with dedifferentiation, enhanced proliferation, invasion, advanced disease and poor prognosis. DLBCL, ALL: high expression is associated with poor prognosis.	KD results in inhibition of proliferation and induction of autophagy. KD results in abrogated lymphomagenesis due to a block in early thymic development.	(Witt et al., 2009b) (Kim and Bae, 2011) (Kristensen et al., 2009) (West and Johnstone, 2014) (Min et al., 2012) (Heideman et al., 2013)
HDAC2 (488 a.a.)	HDAC1, CoREST, NuRD, Sin3, AML1-ETO, PML, PLZF, Bcl6, STAT3, glucocorticoid receptor, YY-1	Negatively regulates transcription by being recruited to DNA as a corepressor. Proliferation control Apoptosis	Gastric, prostate, colorectal cancers, DLBCL, ALL: high expression is associated with poor prognosis.	Cervical cancer cells: HDAC2 KD results in differentiation, apoptosis and p53 independent p21 expression. Genetic HDAC2 mutation reduces intestinal tumor development in APC mice in vivo. KD results in abrogated lymphomagenesis due to a block in early thymic development. CLL cells: KD sensitizes for TRAIL-apoptosis	(Witt et al., 2009b) (Kim and Bae, 2011) (Kristensen et al., 2009) (West and Johnstone, 2014) (Lee et al., 2014)
HDAC3 (428 a.a.)	HDAC4, HDAC5, HDAC7, NCoR/SMRT, AML1-ETO, PML, PLZF, PML-RARα, PLZF-RARα, Bcl6, STAT1, TAT3, GATA1, GATA2, NF-κB, RelA, MEF2D, YY-1, SHP	Represses transcription, binds to TF. Proliferation Differentiation	Gastric, prostate, colorectal cancers: high expression associated with poor prognosis.	APL cells: HDAC3 associated with PML-RARα fusion protein, KD induces differentiation genes. AML: AML-1-ETO binds HDAC3 (and HDACs 1, 2), disrupts cell cycle.	(Witt et al., 2009b) (Kristensen et al., 2009) (Kim and Bae, 2011) (West and Johnstone, 2014)

HDAC8 (377 a.a.)	nd	Proliferation Differentiation	Childhood neuroblastoma: high HDAC8 expression significantly correlates with advanced stage disease, clinical and genetic risk factors and poor long term survival.	Neuroblastoma cells: HDAC8 KD induces differentiation, cell cycle arrest and inhibits clonogenic growth. Lung, colon, cervical cancer cells: KD of HDAC8 reduces proliferation. HDAC8 specific inhibitor selectively induces apoptosis in T-cell derived lymphoma and leukemic cells.	(Witt et al., 2009b) (Kim and Bae, 2011) (Kristensen et al., 2009) (West and Johnstone, 2014)
Class IIa (homologous to Hda1 yeast protein, shuttle between nucleus and cytoplasm, tissue-restricted expression, co-factor for activity - Zn2+)					
HDAC4 (1084 a.a.)	HDAC3-NCoR, GATA1, GCMa, HP-1	Differentiation Angiogenesis	ALL: high expression is associated with poor prognosis, high initial leukocyte count, T-cell ALL and prednisone poor-response.	APL cells: HDAC4 interacts with PLZF-RARα fusion protein, represses differentiation genes. Renal carcinoma cells: KD inhibits expression and functional activity of HIF-1a.	(Witt et al., 2009b) (Kim and Bae, 2011) (Kristensen et al., 2009) (West and Johnstone, 2014) (Gruhn et al., 2013)
HDAC5 (1122 a.a.)	HDAC3-NCoR, GATA1, GATA2, Smad7, HP-1, GCMa	Differentiation	Up-regulated in colorectal cancer. Downregulated in AML and lung cancer.	Erythroleukemia: HDAC5 shuttles from nucleus to cytoplasm upon differentiation, interacts with GATA-1. KD decreased medullablastoma cell growth and viability.	(Witt et al., 2009b) (Kim and Bae, 2011) (Kristensen et al., 2009) (West and Johnstone, 2014)
HDAC7 (855 a.a.)	HDAC3-NCoR, ERα, FLAG1 and 2	Angiogenesis	Up-regulated in colorectal cancer and ALL. Downregulated in lung cancer.	Endothelial cells: HDAC7 silencing alters morphology, migration and tube-forming capacity. KD induced growth arrest in colon and breast cancer cells.	(Witt et al., 2009b) (Kim and Bae, 2011) (Kristensen et al., 2009) (West and Johnstone, 2014)
HDAC9 (1011 a.a.)	nd		Overexpressed in ALL and medulloblastoma, associated with poor prognosis.	KD of HDAC9/10 inhibited homologous recombination and increased sensitivity to DNA damage and decreased medullablastoma cell growth and viability	(Witt et al., 2009b) (Kim and Bae, 2011) (Kristensen et al., 2009) (West and Johnstone, 2014)

Class IIb (homologous to yeast protein Hda1, mostly cytoplasmic location, tissue-restricted expression, co-factor for activity - Zn2+)					
HDAC6 (1215 a.a.)	HDAC11, α-Tubulin, HSP90, SHP, Smad7	Regulation of protein degradation both via the aggresome (a structure that forms in response to misfolded proteins) and the regulation of Hsp90 chaperone activity. Angiogenesis Migration	Oral squamous cell cancer: high expression, increased in advanced stage. Upregulated in breast cancer and CTCL. In breast cancer is associated with enhanced prognosis. Down-regulated in lung cancer.	K562 leukemic cells: targeted inhibition of HDAC6 leads to acetylation of HSP90 and disruption of its chaperone function, resulting in depletion of pro-growth and pro-survival client proteins including the Bcr-Abl oncoprotein. Colon carcinoma cells: HDAC6 targeting blocks EGF induced nuclear translocation of ß-catenin and c-myc expression. KD of HDAC6 causes donwregulation of HIF-1a, VEGFR1/2. HDAC6 involved in TGFβ induced epithelial-mesenchymal transition of lung carcinoma cells.	(Witt et al., 2009b) (Kim and Bae, 2011) (Kristensen et al., 2009) (West and Johnstone, 2014)
HDAC10 (669 a.a.)	HSP90	Angiogenesis	Overexpressed in hepatocellular carcinoma. Poor prognostic indicator in lung cancer.	KD of HDAC10 downregulates VEGFR.	(New et al., 2012) (Kristensen et al., 2009) (West and Johnstone, 2014)
Class III or sirtuins (Silent information regulator 2 family gemology, co-factor for activity - NAD+, conventional HDACis do not affect them)					
SIRT1 (747 a.a.) (Nucleus)	H3K9, H3K14, H3K56, H4K16, H1K26, p53, KU70, Foxo3a, BCL6	Chromatin organization, DNA repair/genome stability, stress, cancer	Upregualted in human lung cancer, prostate cancer and leukemia and has been found downregulated in colon tumors.		(Seto and Yoshida, 2014) (Heltweg et al., 2006) (Ropero and Esteller, 2007)
SIRT2 (352 a.a.) (Cytoplasm)	H4K16, H3K56	Chromatin condensation, mitosis, DNA repair, cancer	Frequently downregulated in human gliomas.		(Seto and Yoshida, 2014) (Ropero and Esteller, 2007)

SIRT3 (399 a.a.) (Nucleus and mitochondria)	H4K16	Chromatin silencing, DNA repair, cellular stress			(Seto and Yoshida, 2014)
SIRT4 (310 a.a.) (Mitochondria)	None				(Seto and Yoshida, 2014)
SIRT5 (314 a.a.) (Mitochondria)	None				(Seto and Yoshida, 2014)
SIRT6 (355 a.a.) (Nucleus)	H3K9, H3K56	Telomeric chromatin / senescence, DNA repair/genome stability			(Seto and Yoshida, 2014)
SIRT7 (400 a.a.) (Nucleus)	H3K18	Cellular transformation			(Seto and Yoshida, 2014)
Class IV (Unknown yeast protein homology, cytoplasmic location, tissue-restricted expression, co-factor for activity - Zn2+)					
HDAC11 (347 a.a.)	HDAC6	Regulates the protein stability of DNA replication factor CDT1 and the expression of interleukin-10	Overexpressed in breast, renal and liver cancer. ALL: high expression is associated with poor prognosis.	It has been implicated in immune system regulation via its role in interleukin-10 expression and OX40L surface expression in Hodgkin lymphoma	(Witt et al., 2009b) (New et al., 2012) (Kim and Bae, 2011) (Kristensen et al., 2009) (Glozak and Seto, 2009) (West and Johnstone, 2014)

KD – knockdown. ALL - acute lymphoblastic leukemia. AML - acute myeloid leukemia. APL - acute promyelocytic leukemia. CLL - chronic lymphocytic leukemia. DLBCL - diffuse large B-cell lymphoma. TF – transcription factor.

proteins sensitive to regulation by acetylation have been identified (Choudhary et al., 2009); (Kim and Bae, 2011). Key cell survival proteins such as p53, Ku70, BCL6, tubulin turn out to be targets for classical HDACs and sirtuins (Bereshchenko et al., 2002); (Gu and Roeder, 1997); (Matsuyama et al., 2002). Therefore, altered HDAC activity may influence both gene expression and other cellular processes including tumor cell apoptosis, growth arrest, differentiation, inhibition of angiogenesis, etc (West and Johnstone, 2014).

d. Control over HATs and HDACs activity

HAT and HDAC activity in mammalian cells is regulated through three main mechanisms: regulation of the enzyme amount, its enzymatic activity, or its availability for interaction with specific transcription factors.

First and the most obvious way of enzyme regulation is the control over their gene expression. For example, *HDAC1* expression is induced by histone hyperacetylation in its promoter, thus creating a feedback loop control (Hauser et al., 2002). Second, their half-life which defines intracellular quantity of a protein. Thus, degradation of Tip60 HAT, involved in apoptosis and DNA double-strand break repair, is regulated via ubiquitination by the ubiquitin ligase Mdm2 (Legube et al., 2002). The third main mechanism that regulates the enzymatic activity of HATs and HDACs involves post-translational modifications and protein–protein interactions. Apart from that, the metabolic cofactors availability might also influence acetylation levels.

Post-translational modifications. Phosphorylation is one of the most common modifications which regulates activity of many HATs and HDACs. For instance, cyclin E/cyclin-dependent kinase 2 phosphorylates CREB-binding protein (CBP) in order to stimulate its HAT activity

which is required for progression to the S-phase of the cell cycle (Ait-Si-Ali et al., 1998). Another example is reduction of HDAC4 activity by sumoylation with E3 ligase RANBP2 (Kirsh et al., 2002).

Protein-protein interactions. Both HATs and HDACs normally work as a part of large, multimolecular complexes. Notably, factors which are not *bona fide* components of the complex were usually described to control HAT activity (Legube and Trouche, 2003).

Third documented mechanism of HATs or HDACs regulation includes either their capacity to interact with specific transcription factors or their subcellular localization (Zanger et al., 2001), (Baek et al., 2002).

5. Relationship between levels of epigenetic regulation

Chromatin architecture and gene expression is regulated by tight cooperation between different epigenetic modifications. Extensive cross-talk between histone and DNA modifications is mediated by several epigenetic adaptors, including chromatin remodeling enzymes, methyl-DNA binding proteins and siRNA. Epigenetic marks are much more than simple on/off switch of gene promoters. An increasing body of evidence indicates a high level of their functional integration.

For a long time, DNA methylation was thought to affect gene expression by mostly modulating the interaction between transcription factors and DNA (Becker et al., 1987), (Iguchi-Ariga and Schaffner, 1989). However, recent discoveries opened another view on downstream effects of DNA methylation, giving the principal role to indirect regulatory mechanisms, where methyl-

DNA binding proteins take the leading position (Ng and Bird, 1999). Moreover, these proteins also serve as general adaptors between DNA methylation and epigenetic histone modifications.

The first example of an adaptor between distinct epigenetic modifications was methyl-DNA binding protein MeCP2. It specifically binds methylated DNA and then recruits histone deacetylase complexes (Jones et al., 1998). Thus, MeCP2 serves as an important factor in the establishment of repressive chromatin state at methylated loci. Since then, several other methyl-DNA binding proteins have been characterized (Hendrich and Bird, 1998). Interestingly, proteins containing both, a methyl-DNA binding domain and a putative histone methyl transferase domain, have also been identified (Kouzarides, 2002). Discovery of these proteins permitted to better understand long-range effects of DNA methylation and its correlation with histone deacetylation and gene silencing.

Experiments on plants and flies showed functional interaction between H3K9 methylation and DNA methylation. In this case, methylation of H3K9 is required for *de novo* DNA-methylation, while the opposite is possible too: histone methylation can be controlled by DNA methylation (Soppe et al., 2002), (Tamaru and Selker, 2001).

Various lines of evidence also suggest a connection between RNAi and DNA methylation. Mutation in *ARGONAUTE4* gene in *Arabidopsis*, which is involved in siRNA processing, affected DNA methylation patterns and altered histone H3-K9 methylation (Zilberman et al., 2003). However, the exact mechanistic basis of the interaction between siRNAs and other epigenetic factors has not yet been determined.

siRNA-mediated suppression of transcription has been reported a number of times to be associated with histone and DNA methylation in genes promoters of mammalian cells (Morris

et al., 2004), (Castanotto et al., 2005), (Suzuki et al., 2005). At the same time, it has been shown that double-stranded RNAs (dsRNAs), targeted to selected promoter regions of the human genes, did not change their DNA methylation state, but provoked histone demethylation, especially H3m2K4 (Li et al., 2006), (Chen et al., 2008). As a result the targeted genes underwent long-lasting activation: the phenomenon termed as RNA activation (RNAa).

Thus, interactions between DNA methylation and histone modifications can be established, maintained and reinforced by involving siRNAs and adaptor molecules, like methyl-DNA binding proteins. In this scenario, many types of interactions are conceivable that would reinforce the stability of epigenetic information (**Figure 10**).

In recent times, after collecting and analyzing an increasing amount of epigenetic data, scientists started coming to the conclusion that local changes in gene expression are rather secondary consequences of epigenetic regulation, whereas the primary outcome is changes in higher-order chromosome organization (for review see (Weissmann and Lyko, 2003)).

The most illustrative examples include studies of affected enzymes such as DNA-methyltransferases or histone deacetylases. It has been revealed that inhibition of their catalytic activity (either by mutations or inhibitors) paradoxically does not lead to global changes in gene expression. Instead of that, it leads to misregulation of only about 1% of genes, notably, in both directions - upregulation and downregulation ((Jackson-Grusby et al., 2001) and (Ehrlich et al., 2001) for DMT genes; (Heider et al., 2006) for HDACs inhibition).

Deletions of both histone methyltransferases in mice caused a loss of centromeric H3K9 methylation and a concomitant reduction of chromosome stability (Peters et al., 2001). Disruption of RNAi pathway in fission yeasts reflected at the level of chromosome structure (Hall et al., 2003). Patients with ICF syndrome (immunodeficiency, centromeric instability, facial

anomalies) have lack of DNMT3B DNA methyltransferase activity and consequently decondensed centromeric heterochromatin, chromosome fusions and multiradial chromosomes with up to 12 arms (Smeets et al., 1994).

These and much more experimental data hints at the idea that epigenetic signals regulate higher order chromosome structure and thus affect gene expression patterns, i.e. indirectly, rather than affecting genes on a locus-specific level. In this sense, consequences of

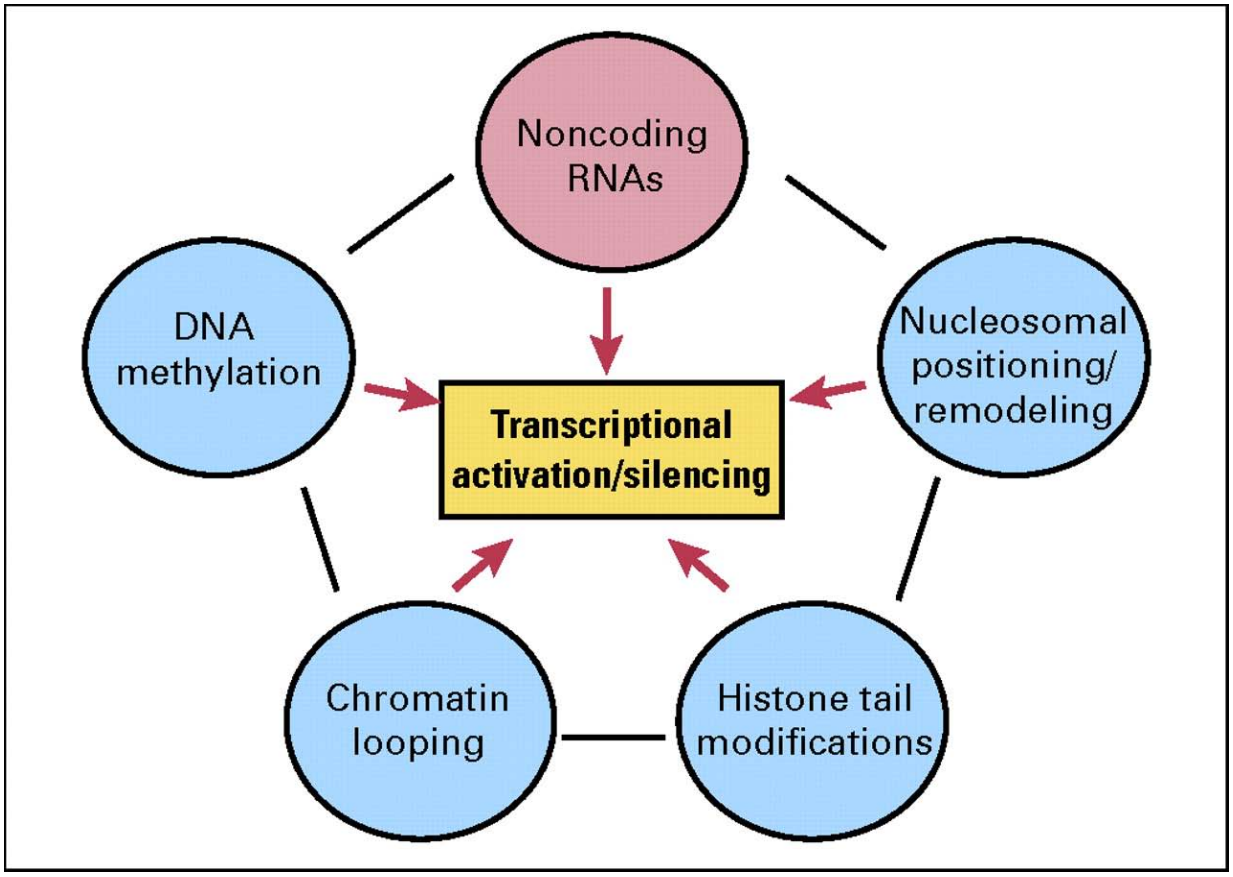


Figure 10. Cooperative interactions in epigenetic regulation.

Epigenetic regulation network involves the DNA methylation, histone modification, chromatin remodeling, ncRNAs, chromatin remodeling and repositioning, which mutually affect each other. The results can be gene silencing or reactivation. [from (van Engeland et al., 2011)]

chromosomal translocations, where entire chromosome loci change their intranuclear locations and hence particular epigenetic environment, gain significance.

6. Epigenetic changes and cancer

Cancer is a genetic disease at the cellular level initiated by alterations in genes from cell proliferation, survival, and other key cellular process pathways (Vogelstein and Kinzler, 2004). Such genes, which have a potential to cause cancer or protect a cell from the path to cancer, are called oncogenes and tumor suppressor genes respectively. In malignant cells oncogenes and/or tumor suppressor genes are modified by mutations or altered at the level of expression by epigenetic modifications. This can occur through chromosome translocations, DNA methylation, histone modifications, miRNA alterations and other processes (Jones and Laird, 1999). Mutations, chromosomal aberrations and global changes in the epigenetic landscape are the hallmarks of cancer.

a. DNA methylation and cancer

Loss of methylation is very often revealed in cancer cells (Jones and Baylin, 2002). If mechanism of DNA methylation is altered, regions of the genome that should be methylated, such as repetitive sequences, may lose stability as a result of the loss of methylation. This genomic instability can then lead to the development of different malignancies. Indeed, the general level of DNA methylation is often decreased in neoplastic cells, causing genetic

instability and unscheduled activation of genes (Feinberg and Vogelstein, 1983). Hypomethylation of repetitive sequences and retrotransposons increases genomic instability by promoting chromosomal rearrangements (Eden et al., 2003), (Howard et al., 2008).

DNA hypomethylation can activate growth-promoting genes, such as *MAGE-1* locus, which is normally expressed only in germ line cells, but is activated in melanoma cells (De Smet et al., 1996), *R-Ras* and *MAPSIN* - in gastric cancer, *S-100* - in colon cancer cells (Wilson et al., 2007).

On the other hand, tumor suppressor genes' promoters (responsible for proliferation, apoptosis, DNA repair, and immortalization) aberrantly gain hypermethylation which typically results in their silencing. Their silencing can therefore promote tumor formation and growth. One of the first discovered examples of such suppression was retinoblastoma (*RB*) gene, whose promoter is found to be methylated in a significant subset of sporadic and even hereditary retinoblastomas (Greger et al., 1989). Methylation silencing of *SFRP* genes (antagonists of the WNT signaling pathway) lead to cell proliferation and promotes early dysplastic colon mucosal lesions in mice. In the majority of prostate cancers, glutathione S-transferase (*GSTP1*) gene, encoding the detoxification enzyme, is often inactivated by hypermethylation.

One of the most possible mechanisms of gene silencing via hypermethylation involves higher activity of DNA methyltransferases in tumor cells. Indeed, altered DNMT activity and expression was observed in numerous diseases including obesity, cardiovascular diseases, type 2 diabetes, autism and cancer, (Milagro et al., 2011), (Chowdhury et al., 2011), (Maier and Olek, 2002), (Grafodatskaya et al., 2010), (Nephew and Huang, 2003). Interestingly, regions

which gain hypermethylation in cancer, are often pre-marked with H3K27me3 polycomb mark in embryonic stem cells (Schlesinger et al., 2007).

b. Histone modifications and cancer

In cancer cells, the balance of histone modification marks is substantially altered. High-throughput sequencing analysis revealed a global loss of H4K16ac and H4K20me3 during tumorigenesis (Fraga et al., 2005).

Chromatin structure and its accessibility for transcription are very regulated by histone modifications. For example, deacetylated histones can block access for transcription factors and therefore prevent the gene expression. Thus, changes in activity of enzymes, responsible for histone acetylation / deacetylation (HATs / HDACs), may alter the transcription of genes controlling cell-cycle progression and developmental events. Indeed, HDACs and HATs are often found overexpressed or altered in another way in various types of cancer (Halkidou et al., 2004), (Song et al., 2005). In leukemia, fusion proteins often involve HAT or HAT-related genes (e.g. MOZ, MORF, CBP and p300) (Yang, 2004). EZH2, the H3K27 HMT, is found overexpressed in breast and prostate cancer (Valk-Lingbeek et al., 2004).

c. Deregulation of miRNAs in cancer

During tumorigenesis, widespread changes in miRNA expression were detected (Lu et al., 2005). miRNAs regulate genes involved in processes which are usually deregulated in cancer,

i.e. transcriptional regulation, cell proliferation and apoptosis. miRNA can be down- or upregulated in cancer cells, depending on their targets. In chronic lymphocytic leukemia, tumor suppressor miRNAs *miR-15* and *16*, that target an antiapoptotic gene *BCL2*, are downregulated. In lung cancer *let-7* is downregulated, which targets the oncogene RAS (Zhang et al., 2007). Opposite examples come from oncogenic miRNAs, which target growth inhibitory pathways. These miRNAs are often upregulated in cancer. Thus, *miR-21* in human glioblastoma (Chan et al., 2005) and *miRNA-155* in breast, lung and several hematopoietic malignancies are upregulated (Kluiver et al., 2006). Several possible mechanisms can be responsible for such changes in miRNA expression, including chromosomal abnormalities and epigenetic alterations (Deng et al., 2008).

d. Translocations and cancer

Chromosome translocations have an important role in the initial steps of carcinogenesis, including leukemia, lymphoma and some solid tumors. Translocations involve non-homologous chromosomes and can be classified as reciprocal (with exchange of segments between two chromosomes) and non-reciprocal (fusion of two acrocentric chromosomes with reduction of chromosomal number).

First suggestion that acquired chromosome abnormalities play a crucial role in the origin of cancer, was conceptualized by Theodor Boveri in 1914 (Boveri, 1914). Technical possibility to prove this theory became available only half a century later. In 1960 with technologies of human chromosome visualization, the association of Philadelphia chromosome with chronic myeloid leukemia (CML) has been discovered by Nowell and Hungerford (Nowell, 1960). Now

it is commonly accepted that many chromosome aberrations are responsible for specific types of malignancies, especially hematological (Rowley, 2001).

Common consequences of chromosome aberrations are the generation of chimeric fusion genes and gain or loss of genetic material. Notably, chromosome translocations frequently lead to changes in constant chromosome positions (Harewood et al., 2010), (Allinne et al., 2014). This event causes relocalisation of genes to the new not typical surroundings, defining an aberrant gene expression in most of the cases. These changes in gene expression, without altering neither any gene structure nor its dosage, can be considered as purely epigenetic. Translocations broadly have two dominating types of consequences: inactivation of tumor suppressor genes and activation or deregulation of proto-oncogenes.

- ***Chimeric genes***

Translocations may fuse the coding sequences of two genes together to generate a chimeric fusion gene, which in turn codes an activated form of the protein affecting the normal cellular physiology (Grignani et al., 1998a), (Minucci et al., 2000), (Lin and Evans, 2000).

Genes, prone to chimer formation, often encode transcription factors regulating differentiation at the cellular, tissue and organismal level. As a result, fusion-gene translocations commonly lead to arrested or aberrant development, or heavy diseases. Thus, fusion-gene translocations are classically recognized as hallmarks of sarcomas and hematopoietic neoplasms.

One of the first studied examples is the Philadelphia chromosome, discovered in patients with chronic myelogenous leukemia (CML) (**Figure 11**). Janet Rowley and colleagues have shown that the Philadelphia chromosome was actually a product of a reciprocal translocation between chromosomes 9 and 22 (Rowley, 1973), which fused the coding sequences of the *BCR* at

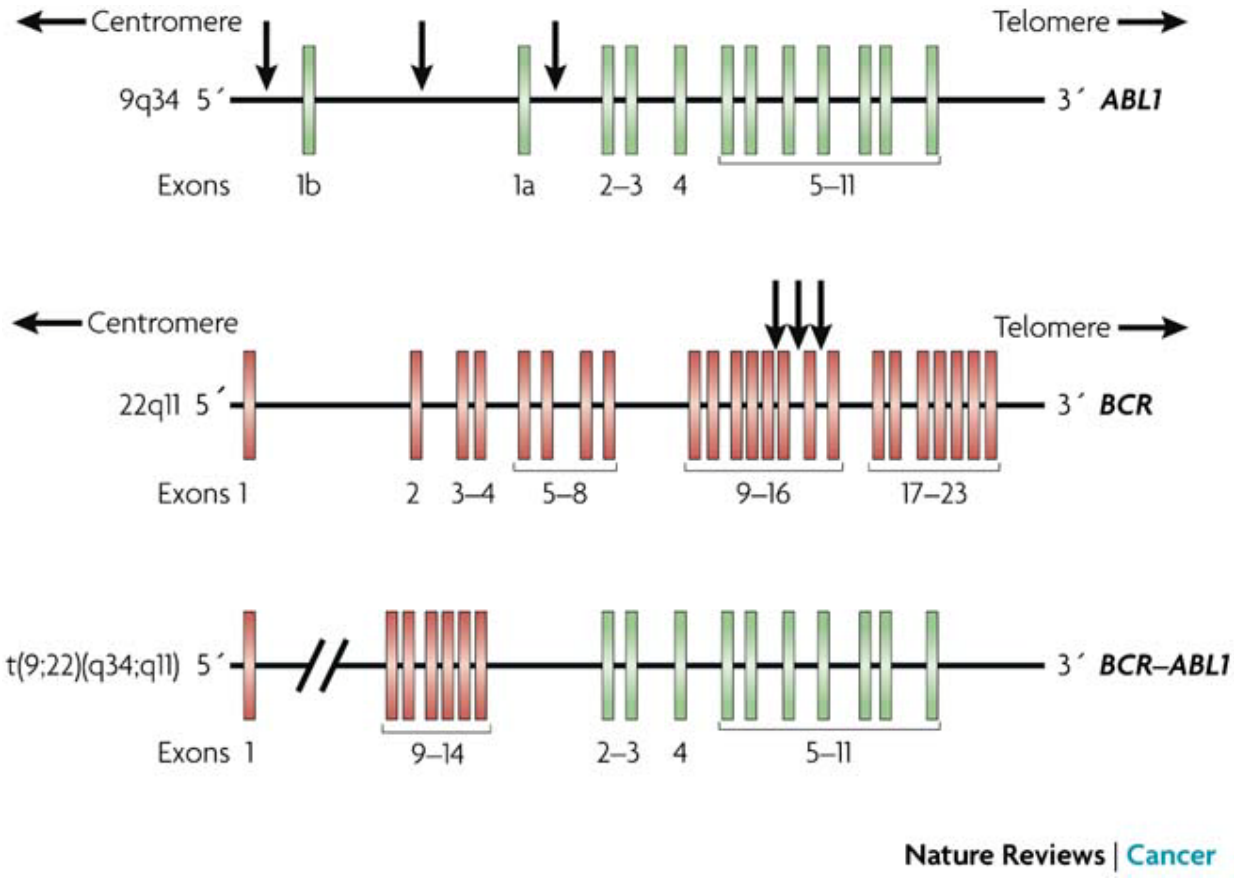


Figure 11. Gene fusion leading to a chimeric gene.

The Philadelphia chromosome, which originates through the translocation t(9;22)(q34;q11), juxtaposes the 5' part of the *BCR* gene at 22q11 with the 3' part of the *ABL1* gene at 9q34, resulting in the creation of a hybrid *BCR-ABL1* fusion gene. The *ABL1* gene is oriented with its 5' end towards the centromere of chromosome 9. The gene spans more than 230 kb and contains two alternative first exons, 1b and 1a, followed by exons 2-11. Exon 1b is located approximately 200 kb upstream of exon 1a. The breakpoints are scattered over a large area (greater than 300 kb) at the 5' end of the gene, either upstream of the first alternative exon 1b, between the two alternative exons, or between exons 1a and 2. Irrespective of the breakpoint location, splicing of the hybrid transcript yields an mRNA in which *BCR* sequences are fused to *ABL1* exon 2. The *BCR* gene has its 5' end towards the centromere of chromosome 22, spans approximately 135 kb and has 23 exons. In most patients with chronic myeloid leukemia, and at least one third of patients with Philadelphia chromosome-positive acute lymphoblastic leukemia, the break occurs in a 5.8 kb major breakpoint cluster region that spans exons 12-16. [from (Mitelman et al., 2007)]

22q11 and *ABL1* genes at 9q34. The BCR-ABL1 fusion protein is a protein tyrosine kinase which constitutively activates cell growth and proliferation signaling pathways. Notably, knowledge of that particular mechanism of CML development, allowed investigators to develop a successful treatment for that disease by specifically inhibiting the fusion BCR-ABL protein's activity.

Another well known fusion protein is TEL-AML1 in acute lymphoblastic leukemia (ALL). TEL-AML1 is a result of t(12;21)(p12–13;q22) translocation, bringing together TEL, a transcription factor of the ETS family, and AML1, a subunit of a core binding factor important in hematopoietic stem cells formation. TEL-AML1 fusion protein causes transcriptional repression of genes by involving histone deacetylases in that process (Pui et al., 2004), (Zelent et al., 2004).

In hematological disorders, 264 gene fusions, involving 238 different genes, have been identified. For the full list see **Table 2**.

- ***Gene dosage***

Some translocations, particularly non-reciprocal ones, may lead to changes in gene dosage. Change in copy number may result in loss of tumor suppressor genes or duplication of oncogenes (Artandi et al., 2000), thus causing cancer formation and progression (Volik et al., 2006), (Frank et al., 2007), or other genomic disorders (Lupski, 1998).

For example, in non-small cell lung cancer, *EGFR* copy number can be higher than normal (Cappuzzo et al., 2005). A higher copy number of *CCL3L1* is associated with lower susceptibility to HIV infection (Gonzalez et al., 2005); a low copy number of *FCGR3B* can increase susceptibility to systemic lupus erythematosus and other inflammatory autoimmune

Table 2. Chromosomal translocations involved in hematopoietic tumors leading to gene fusions.

Type of translocation	Type of tumor	Genes involved	References
t(8;21)(q22;q22)	AML-M2	*AML1/CBFα*(21q22)	Ohki M, Sem. Cancer Biol., 1993;4:369–376
		ETO/MTGβ(8q22)	
t(15;17)(q21;q21)	APL	*PML* (15q21)	Gillard et al., Sem. Cancer Biol., 1993;4:359–368
		RARA (17q21)	
t(8;16)(p11;p13)	*AML	*MOZ*(8p11)	Borrow J et al., Nat Genet., 1996;14:33–41
		CBP(16p13)	
t(9;22)(q34q11.2)	CML/ALL	*BCR*(22q11)	de Klein et al., Nature, 1982;300:765–767
		c-ABL(9q34)	
t(11;17)(q23;q21.1)	AML-M3	*PLZF* (11q23)	Chen et al., EMBO J., 1993;12:1161–1167
		RARA (17q21)	
t(9;11)(p22;q23)	AML-M4, pre-B-ALL	*MLL* (11q23)	Nakamura et al., PNAS, 1993;90: 4631–4635
		AF9 (9p22)	
t(6;11)(q27;q23)	AML-M5, ALL	*MLL* (11q23)	Prasad et al., Cancer Res., 1993;53:5624–5628
		AF6 (6q27)	
t(6;9)(p23;q34)	AML-M1,M2,M4,M5	*DEK* (6p23)	von Lindern, Mol. Cell Biol., 1992;12:1687–1697
		CAN (9q34)	
t(16;21)(p11;q22)	AML	*FUS*(16p11)	Shimizu et al., PNAS, 1993;90:10280–10284
		ERG(21q22)	
t(16;21)(q24;q22)	t-AML, MDS	*AML1*(21q22)*MTG*(16q24)	Gamou T et al., Blood, 1998;91:4028–4037.
t(3;21)(q26;q22)	CML	*AML1*(21q22)	Mitani et al., EMBO J., 1994;13:504–510
		EVI-1(3q26)	
t(3;21)(q26;q22)	Myelo-dysplasia	*AML1*(21q22)	Nucifora et al., PNAS, 1993;90:7784–7788
		EAP(3q26)	
t(7;11)(p15;p15)	AMLM2, M4	*NUP98*(11p15)	Borrow J et al., Nat Genet., 1996;12:159–167
		HOXA9(7p15)	
t(1;11)(q23;p15)	AML-M2	*NUP98*	Nakamura T et al., Blood, 1999;94:741–747
		PMX1	
t(1,11)(p32;q23)	ALL	*MLL/HRX(*11q23)	Bernard et al., Oncogene, 1994;9: 1039–1045
		AFP1(1p32)	
t(17;19)(q22;p13)	Pro-B-ALL	*E2A*(19p13)	Inaba et al., Science, 1992;257:531–534
		HLF(17q22)	
t(12;22)(p13;q11–12)	MDS	*ETV6*(12p13)/TEL	Buijs A et al., Oncogene, 1995;10:1511–1519
		MN1(22q11)	
t(8;22)(p11;q13)	AML-M5	*MOZ*(8p11)	Lai et al., Cancer Genet Cytogenet, 1992;60: 180–182
		P300(22q13)	Chaffanet et al., Genes Chromosomes Cancer, 2000;28: 138–144.
t(5;12)(q33;p13)	CMML	*TEL*(12p13)	Golub et al., Cell, 1994;77: 307–316
	*Nalm-6	*PDGFRβ*(5q33)	Wlodarska et al., Blood, 1997;89: 1716–1722

t(1;19)(q23;p13)	AML-M7, Pre-B-ALL	*OTT* and *MAL*	Nourse et al., Cell, 1990;60:535–545
		E2A(19p13.3)*PBX1*(1q23)	Kamps et al., Cell, 1990;60:547–555
t(12;21)(p12–13;q22)	Pre-B-ALL	*TEL*(12p12)	Romana et al., Blood, 1995;86:4263–4269
		AML1(21q22)	
t(4;11)(q21;q23)	Pre-B-ALL	*MLL*(11q23)	Djabali et al., Nature Genet., 1992;2: 113–118
		AF4(4q21)	Gu et al., Cell, 1992;71:701–708
t(11;19)(q23;p13)	Pre-B-ALL, T-ALL	*MLL*(11q23)	Tkachuk et al., Cell, 1992;71:691–700
		ENL(19p13)	Yamamoto et al., Oncogene, 1993;8: 2617–2625
t(X;11)(q13;q23)	T-ALL	*MLL*(11q23)	Corral et al., PNAS, 1993;90: 8538–8542
		AFX1(Xq13)	
t(2;5)(p23;q35)	ALCL (NHL)	*NPM* (5q35)	Morris et al., Science, 1994;263:1281–1284
		ALK (2p23)	
t(4;16)(q26;p13)	T-lymphoma	*BCM*(16p13.1)*IL2*(4q26)	Laabi et al., EMBO J., 1992;11:3897–3904
t(4;11)(q21;p15)	pre-T-LBL	*NUP98*(11p15.5)	Kalatzis et al., Cancer Genet Cytogenet, 1993;69: 122–125
		RAP1GDS1(4q21)	Mecucci et al., Br J Haematol, 2000;109: 788–793
t(5;14)(q33;q32)	AML	*CEV14*(14q32)	Abe et al., Blood, 1997;90:4271–4277.
		PDGFRB(5q33)	
t(1;22)(p13;q13)	AMKL	*RBM15*(1p13)	Lion et al., Blood, 1992;79:3325–3330.
		MKL(22q13)	Ma et al., Nat Genet., 2001;28: 220–221.
t(10;11)(p13;q21)	pre-T-LBL	*CALM* (11q21)	Chaplin et al., Blood, 1995;85:1435–1441
		AF10(10p13)	

ALCL — anaplastic large-cell lymphoma, NHL — non-Hodgkin's lymphoma, ALL — acute lymphoblastic leukemia, AML — acute myeloid leukemia, APL — acute promyelocytic leukemia, AML — acute monoblastic leukemia, Pre-T-LBL — pre-T cell lymphoblastic leukemia/lymphoma, AMKL — acute megakaryoblastic leukemia, CML — chronic myelogenous leukemia, CMML — chronic myelo-monocytic leukemia, T-PLL — pro-lymphocytic leukemia, CLL — chronic lymphocytic leukemia, FL — follicular lymphoma, DLCL — diffuse large-cell lymphoma, BL — Burkitt's lymphoma, MDS — myelodysplastic syndrome.

* Nalm-6 is a pre-B-cell line, established from the peripheral blood of a 19-year-old man with acute lymphoblastic leukemia (ALL). [from (Nambiar et al., 2008)]

disorders (Aitman et al., 2006). In addition, copy number variation has also been associated with diseases such as autism (Sebat et al., 2007), idiopathic learning disability (Knight et al., 1999) and schizophrenia (Sutrala et al., 2008).

- ***Epigenetic abnormalities***

Juxtaposition of oncogenes and a transcription control element of an active gene on a different chromosome is another common consequence of translocations (Nussenzweig and Nussenzweig, 2010). The juxtaposition as well as mere relocalization of the translocated region in the nuclear space (Harewood et al., 2010), (Allinne et al., 2014) leads to deregulation of important genes, particularly proto-oncogenes and tumor suppressor genes, crucial for regulation of important cellular processes (Korsmeyer, 1992), (Mitelman et al., 2004). Notably, in this case, translocations do not alter any gene structure nor its dosage but still lead to changes in gene expression. Thus, these changes in gene expression might be considered as purely epigenetic.

Oncogene-activating translocations are tightly associated with lymphoid malignancies. The most studied t(14;18) translocation in follicular lymphoma brings *BCL2* gene on chromosome 18 under the control of *IgH* enhancer on chromosome 14. Break on chromosome 14 occurs at JH segments of *IgH* loci, while on the chromosome 18, potential points of translocation are concentrated in the MBR region, 150 bp long (**Figure 12a**). This juxtaposition confers anti-apoptotic qualities to the cell.

Burkitt's lymphoma harbors a translocation involving chromosomes 8 and 14. This process takes place mostly during the class switch recombination of Ig genes, and the break occurs at the switch regions of the immunoglobin heavy chain genes (IgH) segments. The translocation

places fragment of chromosome 8 with proto-oncogene C*MYC* on the 14 chromosome with *IgH* genes. As a result, CMYC protein from the cell proliferation signal pathway is overexpressed in lymphoid cells (**Figure 12b**). The initial theory explained it by influence of the IgH powerful promoter on *CMYC* gene (Dalla-Favera et al., 1982). However the recent discoveries disprove this theory and propose another mechanism which will be discussed later (Allinne et al., 2014).

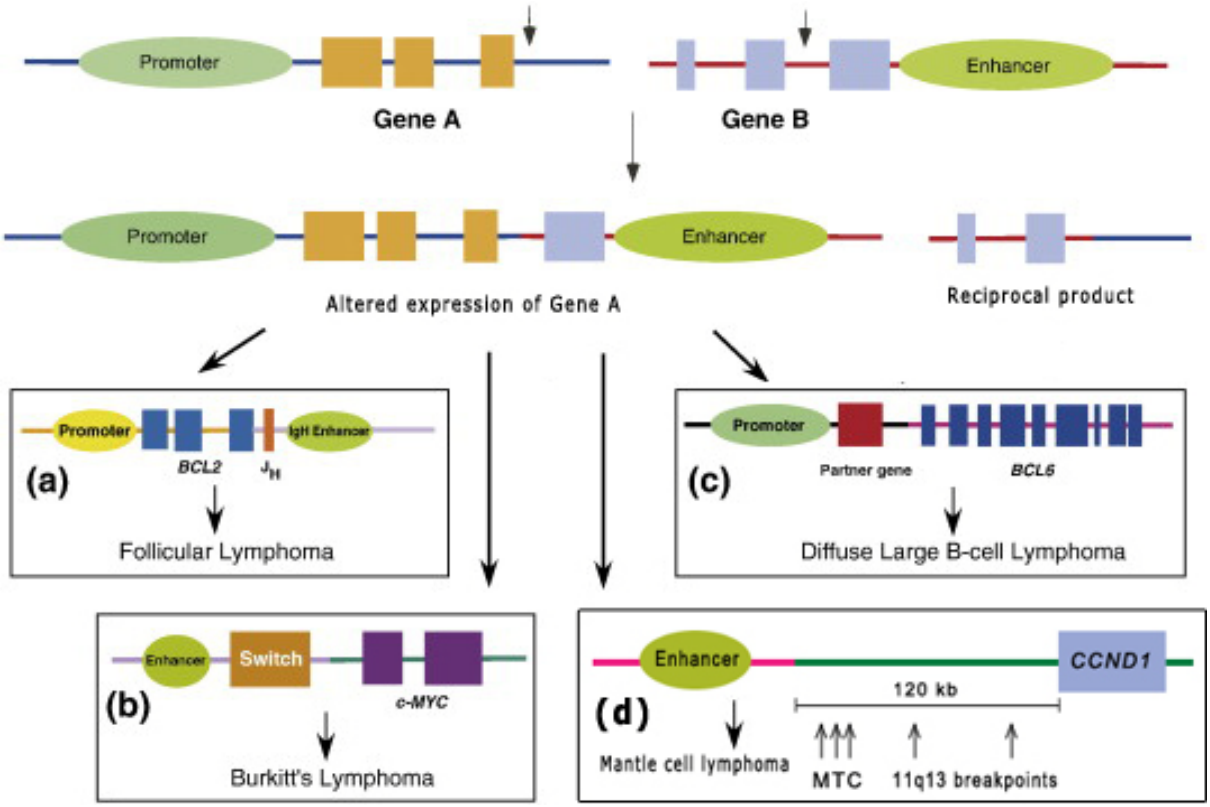


Figure 12. Chromosomal translocations resulting in juxtaposition of promoter/enhancer elements to oncogenes.

In some lymphoma and leukemia, translocations result in juxtaposition of the coding region of a gene (gene A) to enhancer elements of another gene (gene B). This leads to enhanced expression of the gene A under the influence of either the enhancer or alternative promoters. (a) Follicular lymphoma with t(14;18) translocation. (b) Burkitt's lymphoma with t(8;14) translocation. (c) Diffuse large B-cell lymphoma with various translocations involving the BCL6 gene on chromosome 3 and different partner chromosomes. (d) Mantle cell lymphoma with t(11;14) translocation. [with changes from (Nambiar et al., 2008)]

One of the most common genetic abnormalities in diffuse large B-cell lymphoma comprises of various translocations involving the *BCL6* on 3q27 band with one of the partner chromosomes (14q32, 2p11, 22q11, 4p11, 6p21, 11q23) (**Figure 12c**). As a result of these translocations, *BCL6* comes under the influence of a new promoter and is overexpressed (Ohno, 2004).

Another example is mantle cell lymphoma (MCL) with a translocation between 11 and 14 chromosomes juxtaposing Cyclin D1 proto-oncogene with *IgH* promoter (**Figure 12d**). The majority (80-90%) of translocation breakpoints on the 14 chromosome occur at the major translocation cluster (MTC) at an approximate distance of 120 kb from *CCND1* gene (de Boer et al., 1993).

A list of lymphoid malignancies associated with chromosomal translocations is represented in **Table 3**.

Table 3. Chromosomal translocations involved in hematopoietic tumors leading to altered gene expression.

Type of translocation	Affected gene	Type of tumor	Mechanism of activation	References
t(8;14)(q24;q32)	*c-MYC*(8q24)	BL, B-ALL	Relocation to IgH locus	Rabbits et al., Adv Immun, 1991;50:119–146
t(2;8)(p12;q24)	*c-MYC*(8q24)	B-ALL	Relocation to IgH locus	Rabbits et al., Adv Immun, 1991;50:119–146
t(8;22)(q24;q11)	*c-MYC*(8q24)	B-ALL	Relocation to IgH locus	Rabbits et al., Adv Immun, 1991;50:119–146
t(8;14)(q24;q11)	*c-MYC*(8q24)	T-ALL	Relocation to TCR-α locus	Shima et al., PNAS, 1986;83:3439–43
t(8;12)(q24;q22)	*c-MYC*(8q24)	B-CLL, ALL	Relocation to Ig locus	Rimokh et al., Genes Chrom. Cancer, 1991;3:24–36
	BTG(12q22)			
t(7;19)(q35;p13)	*LYL1*(19p13)	T-ALL	Relocation to TCR-β locus	Mellentin et al., Cell, 1989;58: 77–83
t(1;14)(p32;q11)	*TAL1/SCL*(1p32)	T-ALL	Relocation to TCR-α Locus	Baer et al., Sem. Cancer Biol., 1993;4:341–347
t(7;9)(q35;q34)	*TAL2*(9q34)	T-ALL	Relocation to TCR-β locus	Baer et al., Sem. Cancer Biol., 1993;4:341–347
t(11;14)(p15;q11)	*RBTN1/TTG1*(11p15)	T-ALL	Relocation to TCR-δ Locus	Boehm et al., EMBO J, 1988;7:385–94
				McGuire et al., Mol. Cell. Biol, 1989;9:2124–32
				Boehm et al., PNAS, USA, 1991;88:4367–71
t(11;14)(p13;q11)	*RBTN2/TTG2*(11p13)	T-ALL	Relocation to TCR-δ/α/β locus	Cheng et al., J Exp Med, 1990;171:489–501
				Yoffe et al., Blood, 1989;74:374–9
				Foroni et al., Genes Chr Cancer, 1990;1:301–9
				Garcia et al., Oncogene, 1991;6:577–82
t(7;11)(q35;p13)	*RBTN2/TTG2*(11p13)	T-ALL	Relocation to TCR-δ/α/β locus	Sanchez-Garcia et al., Sem. Cancer Biol., 1993;4:349–358
t(10;14)(q24;q11)	*HOX11*(10q24)	T-ALL	Relocation to TCR-α/β locus	Lu et al., Genes Chr Cancer, 1990;2:217–22
				Kagan et al., PNAS, USA, 1989;86:4161–5
				Zutter et al., PNAS, USA, 1990;87:3161–5
t(7;10)(q35;q24)	*HOX11*(10q24)	T-ALL	Relocation to TCR-α/β locus	Dube et al., Blood, 1991;78:2996–3003
				Hatano M et al., Science, 1991;253:79–82
t(3;14)(q27;q32)	*BCL6*(3q27)	DLCL, FL	Relocation to IgH locus	Kerckaert et al., Nature Genet., 1993;5:66–70
				Ye et al., Science, 1993;262:747–750

t(14;18)(q32;q21)	*BCL2*(18q21)	FL	Relocation to IgH/IgL locus	Bakshi et al., PNAS, USA, 1987;84:2396–400
				Buchonnet et al., Leukemia, 2002;16: 1852–6
				Cotter et al., Blood, 1990;76:–131–5
				Wyatt et al., J. Exp Med, 1992;175:1575–88
t(11;14)(q13;q32)	*BCL1*(11q13)	B-CLL	Relocation to IgH locus	Tsujimoto et al., Science, 1984;224:1403–6
				Tsujimoto et al., Nature, 1985;315:340–3
				Welzel et al., Cancer Res., 2001;61: 1629–1636
t(10;14)(q24;q32)	*LYT10*(10q24)	B - lymphoma	Relocation to IgH locus	Neri et al., Cell, 1991;67:1075–1087
t(14;19)(q32;q13.1)	*BCL3*(19q13.1)	B-CLL	Relocation to IgH locus	Ohno et al., Cell, 1990;60:991–997
				Wulczyn et al., Nature, 1992;358:597–599
t(5;14)(q31;q32)	*IL-3*(5q31)	pre-B-ALL	Relocation to IgH locus	Grimaldi et al., Blood, 1989;73:2081–2085
				Meeker et al., Blood, 1990;76:285–289
t(7;9)(q34;q34.3)	*TAN1*(9q34.3)	T-ALL	Relocation to TCR-β locus	Ellisen et al., Cell, 1991;66:649–661
				Burnett et al., Genes Chrom. Cancer, 1991;3:461–467
t(1;7)(p34;q34)	*LCK*(1p34)	T-ALL	Relocation to TCR-β Locus	Tycko et al., J. Exp. Med., 1991;174:867–873
t(X;14)(q28;q11)	*C6.1B*(Xq28)	T-PLL	Relocation to TCR-α locus	Stern et al., Oncogene, 1993;8:2475–2483
t(14;21)(q11;q22)	*BHLHB1*(21q22)	pre-T-LBL	Relocation to*TCRA/D* locus	Wang et al., PNAS, 2000;97: 3497–3502
t(1;14)(q21;q32)	*BCL-9*(1q21)	B-ALL and MALT lymphoma	Relocation to IgH locus	Willis et al., Blood, 1998;91:1873–81
				Zhang et al., Nat Genet, 1999;1:63–8

ALCL — anaplastic large-cell lymphoma, NHL — non-Hodgkin's lymphoma, ALL — acute lymphoblastic leukemia, AML — acute myeloid leukemia, APL — acute promyelocytic leukemia, AML — acute monoblastic leukemia, Pre-T-LBL — pre-T cell lymphoblastic leukemia/lymphoma, AMKL — acute megakaryoblastic leukemia, CML — chronic myelogenous leukemia, CMML — chronic myelo-monocytic leukemia, T-PLL — pro-lymphocytic leukemia, CLL — chronic lymphocytic leukemia, FL — follicular lymphoma, DLCL — diffuse large-cell lymphoma, BL — Burkitt's lymphoma, MDS — myelodysplastic syndrome. [from (Nambiar et al., 2008)]

II - Lymphomas

1. Etiology

Lymphoid malignancy is a cancer involving cells of the immune system. Lymphoid malignancies can be generally divided into two major groups: lymphomas and leukemias. Lymphoma is a malignant transformation of white blood cells usually in a lymph node or occasionally in another organ. Leukemia is an increased and uncontrolled production of immature and abnormal leucocytes in the bone marrow and other blood-forming organs.

Lymphoma is divided into the two main types: Hodgkin's (HL, about 10%) and non-Hodgkin's lymphoma (NHL, about 90% of cases) (Shankland et al., 2012). The first type usually consists of an abnormal type of B lymphocyte, named Reed-Sternberg cells, which do not undergo hypermutation to express their antibody. In the non-Hodgkin's lymphoma, both types of lymphocyte, B-cells and T-cells, can be affected. Lymphomas can be subdivided into over 35 different subtypes.

50% of the B-cell NHLs are comprised of small B-cell lymphomas, which includes follicular lymphoma (FL; 40%), mantle cell lymphoma (MCL; 3%– 4%), and B-cell chronic lymphocytic leukemia (B-CLL; 3%– 4%) are included (**Figure 13**). The majority of the remaining 50% of B-cell NHLs are represented by diffuse large B-cell lymphoma (DLBCL), subdivided into germinal

center (GC) B-cell–like (GCB-DLBCL) or activated B-cell–like (ABC-DLBCL) (Taylor et al., 2013b).

Lymphomas, as well as leukemias, represent an interesting class of cancers where almost 100% of malignant transformations are caused by chromosomal translocations (for review see (Nussenzweig and Nussenzweig, 2010), (Hassler et al., 2013)) (**Table 3**). During maturation, lymphocytes undergo the programmed genomic alterations such as: rearrangement of VDJ cell receptors genes and somatic hypermutation. Mistakes in these processes lead to chromosome translocations, a cytogenetic hallmark of most part of lymphomas (Alt et al., 2013). Clear examples of such mistakes are translocations involving chromosome 14 with immunoglobulin heavy-chain (*IgH*) locus and *BCL2* gene on 18 chromosome in case of follicular lymphoma, *CMYC* gene on 8 chromosome in Burkitt's lymphoma, *CCND1* gene on 11 chromosome in mantle cell lymphoma (Shaffer et al., 2002), (Liu et al., 2004).

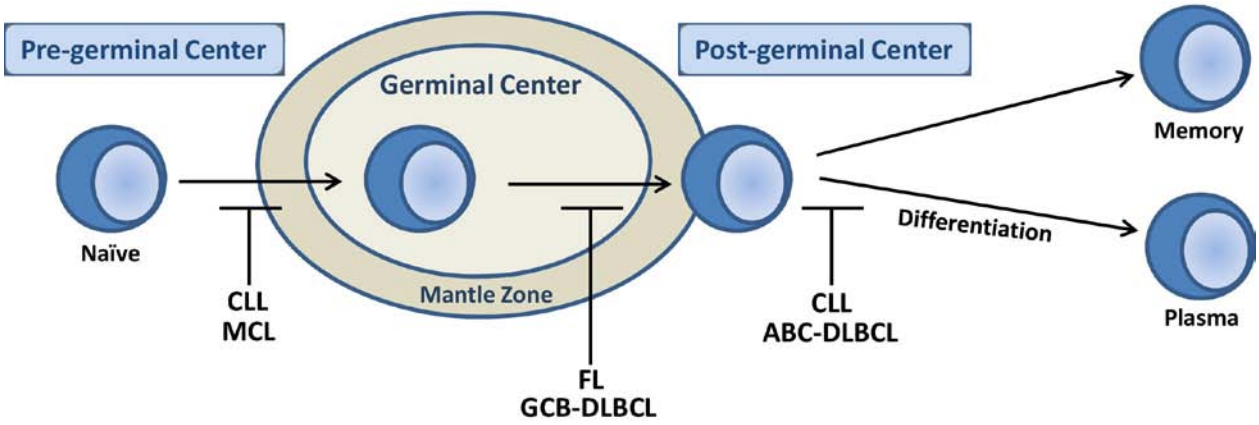


Figure 13. B-cell malignancies and their normal counterparts.

Epigenetic alterations with oncogenic potential may occur at any stage of normal B-cell development and lead to an accumulation of cells at specific stages of differentiation. Abbreviations: CLL, chronic lymphocytic leukemia; MCL, mantle cell lymphoma; FL, follicular lymphoma; GCB-DLBCL, germinal center B-cell-like diffuse large B-cell lymphoma; ABC-DLBCL, activated B-cell-like diffuse large B-cell lymphoma. [from (Taylor et al., 2013b)]

2. Epigenetics of lymphomas

Development of lymphoid malignancies involves a variety of mechanisms which in one way or another affect epigenetic and transcriptional modifiers, which appears to be a recognizable feature of B-cell lymphomas (Shaknovich and Melnick, 2011).

a. Aberrant DNA methylation patterning

One of the important functions of DNA methylation is to maintain repetitive sequences of the genome in a stable state. If this mechanism is altered it leads to genomic instability and hence to development of different malignancies (Goelz et al., 1985). Extensive studies of DNA methylation have revealed hundreds of individual genes aberrantly methylated in different lymphomas and leukaemias (reviewed in (Taylor et al., 2013a); (Hatzimichael and Crook, 2013)).

Apart from the fact that DNA methylation patterns of genome are dramatically affected in cancer cells, it has become recently evident that DNA methyltransferases and MBD proteins are often altered too, thus contributing to development of human malignancies. For instance, up to 25% of patients with acute myeloid leukaemia (AML) harbor recurrent mutations in *DNMT3A* gene (Ley et al., 2010). *DNMT3B* gene is downregulated in CLL (Kn et al., 2004). *DNMT1*, *DNMT3A* and *DNMT3B* are overexpressed in DLBCL (Amara et al., 2010).

b. Disequilibrium of histone modification marks

During normal B-cell development, histone modifications are responsible for maintaining the heritable transcriptional states and lineage fidelity (Parra, 2009). Altered activity of histone modifying enzymes leads to disturbed balance in chromatin modifications, which can affect gene expression of pro-apoptotic genes and proto-oncogenes which contributes to the lymphomagenesis. Indeed, the histone acetylation level is perturbed in different types of cancer, and global histone modification levels are predictive of cancer recurrence (Seligson et al., 2005).

- ***Histone methylation***

Histone-modifying enzymes are frequently targeted by somatic mutations in B-cell lymphoma. Thus, mixed lineage leukaemia (MLL) family of histone methyltransferases (known to mediate H3K4 trimethylation) are frequently mutated or duplicated in acute leukaemias (Rege-Cambrin et al., 2005) and NHL (Morin et al., 2011). A fusion form of that protein is known to activate oncogenic target genes such as *HOXA9* and *EVI1* (Mills, 2010).

EZH2, a component of the Polycomb repressive complex (PRC), methylates histone 3 lysine 27 (H3K27). During early B-cell development *EZH2* is required for VDJ recombination (Su et al., 2003). In mature B-cells it was shown to be downregulated, but after T-cell dependent activation in germinal center (GC), it is strongly expressed (Velichutina et al., 2010). *EZH2* was reported to be over-expressed in several types of leukaemia (Simon and Lange, 2008) and B-cell lymphoma (Sneeringer et al., 2010), and to be mutated in some myeloid malignancies (Ernst et al., 2010), (Nikoloski et al., 2010).

The frequent occurrence of defective MLL2 and over-expressed EZH2 underscores the significance of H3K4me3 and H3K27me3 in aberrant epigenetic programming. Cytogenetic studies of various cancer genomes have demonstrated recurrent translocations and coding mutations in other histone lysine methyltransferases like MMSET (Martinez-Garcia et al., 2011), UTX (van Haaften et al., 2009), and in demethylases, like JMJD2C (Rui et al., 2010).

- ***Histone acetylation***

Histone acetyltransferases and histone demethylases have broad cellular activities: they regulate chromatin structure and activity of vast majority of non-histone proteins. At least 1750 proteins in addition to histones have been shown to be acetylated in leukemia cells (Chi et al., 2010).

Decreased HAT activity caused by gene deletions and somatic mutations, together with gain-of-function mutations of HMTs, leads to repressed chromatin states linked to malignant processes in lymphoid malignancies (Hassler et al., 2013). Two highly related HATs, CBP and p300, often harbor mutations in their HAT coding domain in follicular and diffuse large B-cell lymphoma (Pasqualucci et al., 2011). The same study pointed at the importance of allelic dosage of HAT genes. In B-cell lymphomas, CBP and p300 are known for acetylating proteins involved in lymphomagenesis such as nuclear factor kB (NFkB), p53, BCL6 and Hsp90 (Cerchietti et al., 2010).

There are several examples of translocations involving HATs such as MLL-CBP and MOZ-TIF2; altered expression of HAT genes is observed in a range of cancers (reviewed in (Dawson and Kouzarides, 2012)).

Abnormal histone deacetylases (HDACs) play a key role in many human diseases. Class I and Class II HDACs were implicated in several types of cancer, including lymphomas (reviewed in (Witt et al., 2009a)).

Activity of HDACs is very often altered in lymphoid malignancies, whether by overexpression, mutations or by being involved in the complex with fusion oncoproteins. Chimeric fusion proteins, such as PML-RARa, PLZF-RARα, and AML1-ETO, the result of chromosomal translocations in some leukemia cases, usually recruit HDACs for their activity leading to gene silencing (Grignani et al., 1998b), (Dawson and Kouzarides, 2012), (Cress and Seto, 2000). The expression of different HDACs itself has been observed to be upregulated in various types of cancer (West and Johnstone, 2014), (Kim and Bae, 2011). For example, chronic lymphocytic leukemia is characterized with generally increased HDAC expression (Yang et al., 2015). Although mutations in HDACs are relatively rare, mutations in HDAC2 and HDAC4 were reported in human epithelial cancer cell lines and breast cancer, respectively (New et al., 2012).

3. Mantle Cell Lymphoma

Mantle cell lymphoma (MCL) is a malignant proliferation of B-cells in the mantle zone of lymphoid follicles that have a striking tendency to disseminate throughout the body, infiltrating the lymphoid tissues, bone marrow, peripheral blood, extranodal sites and gut (Fisher et al., 1995). It is a rare and relatively aggressive disease accounting for 5–7% of non-Hodgkin lymphomas in adults (Schmidt and Dreyling, 2008). MCL can occur at any time between the

late 30s to older ages, but it is more common over 50. It is three fold more frequent in males than in females. The clinical course is often indolent or moderately aggressive at diagnosis, with few or no symptoms. Nevertheless, with time, the disease invariably becomes clinically aggressive with frequent remissions (60%-90%) after chemotherapy, showing the worst long-term survival among all B-cell lymphoma subtypes (Zucca et al., 1995). The median survival of diagnosed patients is 3–5 years (Fisher, 2005). MCL cells express CD5+, CD19+, CD20+, CD22+, CD24+, CD43+, CD79a+ B-cell antigens, and are usually CD10 and CD23 negative (Bertoni et al., 2004).

Cytogenetic analyses have revealed that MCL is closely associated with a t(11;14)(q13;q32) translocation, juxtaposing the immunoglobulin heavy chain (*IgH*) locus on chromosome 14 with the *BCL-1* locus in early B-cells (**Figure 12d**). Translocation t(11;14), apart from mantle cell lymphoma, can be found in some cases of diffuse large cell lymphoma, chronic lymphocytic leukemia and multiple myeloma. In addition to Cyclin D1 overexpression, supplementary genetic alterations appear to disturb the cell cycle machinery, interfere with the cellular response to DNA damage and disruption of (Rizzatti et al., 2005).

Translocation (11;14) leads to overexpression of a number of genes, including the cell cycle regulating factor - cyclin D1 (*CCND1*). This functions as a permanent “on switch” committing the cell to complete the cycle (Bosch et al., 1994), (Dreyling et al., 1997) (**Figure 14)**. However, there exist a small number of cases that express cyclin D2 or D3 instead of cyclin D1 (Wlodarska et al., 2008).

Human *IgH* locus has several powerful enhancers (**Figure 14**). The *IgH* intronic enhancer (Eμ) is located between the constant (C_H) and the joining (J_H) regions. It is involved in VDJ rearrangement and gene expression in early B-lineage cells (Chen et al., 1993). The *IgH* 3′-

enhancers are located 25 kb downstream of the Cα gene. The 3′-enhancers consist of four DNase I-hypersensitive sites: HS1, 2, 3 and 4. The initial hypothesis suggested Eμ and 3′-enhancers being responsible for *CCND1* overexpression in case of mantle cell lymphoma (Wang and Boxer, 2005). However, our findings indicated another mechanism, which will be discussed below.

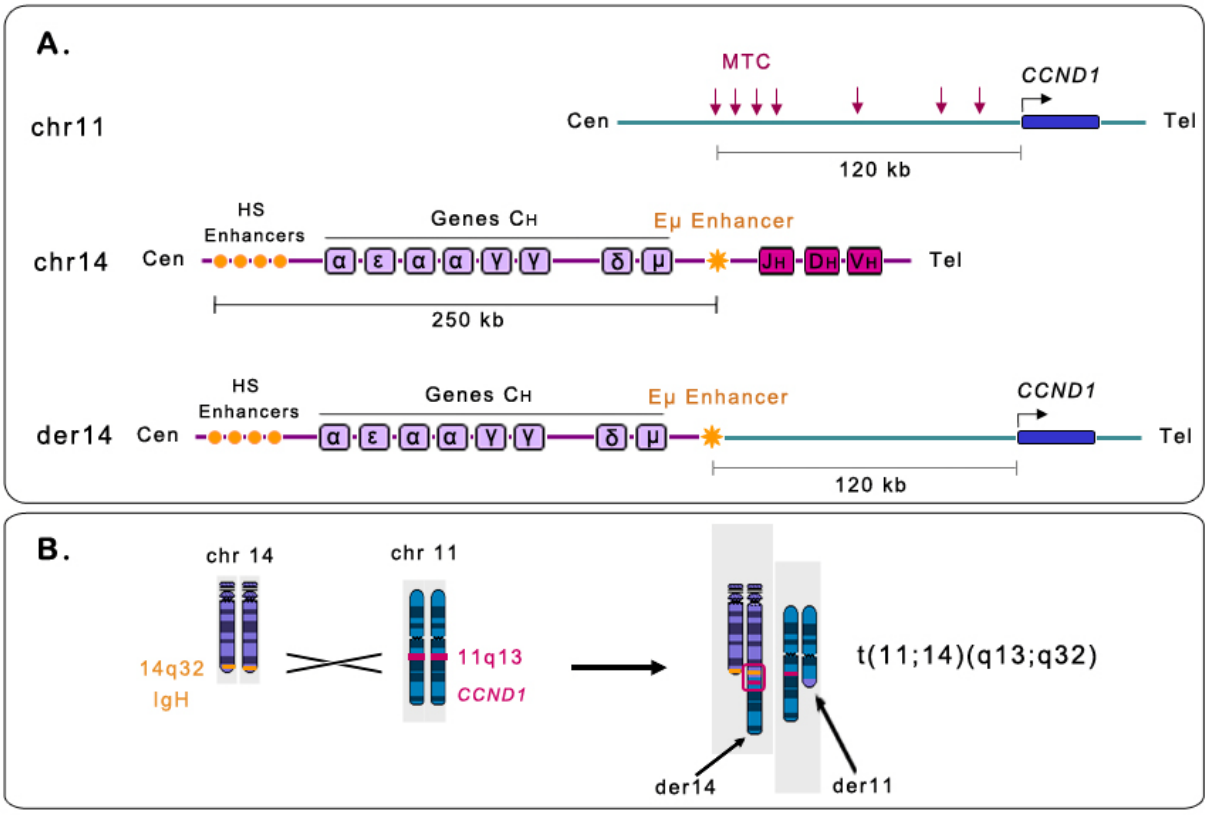


Figure 14. Translocation t(11;14)(q13;q32) in mantle-cell lymphoma.

A. Genomic structure of the Cyclin D1 locus on chromosome 11q13. Most of the breakpoints occur at the major translocation cluster (MTC). Usually the distance between the break and *CCND1* gene is about 120 kb. Normal immunoglobulin heavy chain (IgH) locus on chromosome 14q32 displaying the genomic structure of the constant (C_H), joining (J_H), diversity (D_H), and variable (V_H) region. The Eμ enhancer is located between the constant and the joining regions. **B.** As a result of the t(11;14)(q13;q32) translocation, Cyclin D1 is brought under the control of the Eμ IgH enhancer on the derivative of 14 chromosome.

Translocation breakpoints in 11q13 locus occurring in MCL are scattered over an area spanning more than 100 kb. However, a vast majority of these are located within a ~100 bp segment, called the major translocation cluster (MTC) (Williams et al., 1993). MTC is located ~120 kb upstream (centromeric) of the *CCND1* gene. Ten to twenty percent of MCL cases may harbor two minor translocation clusters, approximately 22kb from the MTC towards the telomere (Rimokh et al., 1993).

Cyclins are cell cycle regulating proteins that increase their quantity during cell cycle progression and degrade in mitosis (Sewing et al., 1993). Cyclins D regulate transition from G1 to S phase. Cyclin D1 protein functions in complex with cyclin dependent kinases CDK 4/6. This complex phosphorylates the retinoblastoma (RB) protein. As a result, RB loses its growth suppressive effects and the cell is promoted from G1 to S face in the cell cycle (Gladden and Diehl, 2003). Another mechanism involves CDK inhibitors p27kip1, p21 and cyclin E/CDK2 complexes (**Figure 15**). Besides that, D type cyclins have their CDK independent functions. In cancer cells, Cyclin D1 may interact with CCAAT enhancer binding protein alpha (CEBPα) and thus serve as a DNA binding protein (Ewen and Lamb, 2004). In normal hematopoietic cells, Cyclin D1 is not expressed, whereas Cyclin D2 and D3 are expressed in both normal and neoplastic B and T lymphocytes. Expression of Cyclin D1 is observed in hematologic malignancies, like MCL and multiple myeloma (MM). Notably, in some cases of MM and hairy cell leukemia (HCL), Cyclin D1 expression has been reported without t(11;14) translocation, but with extra copies of chromosome 11 or a hyperdiploid phenotype (Troussard et al., 2000).

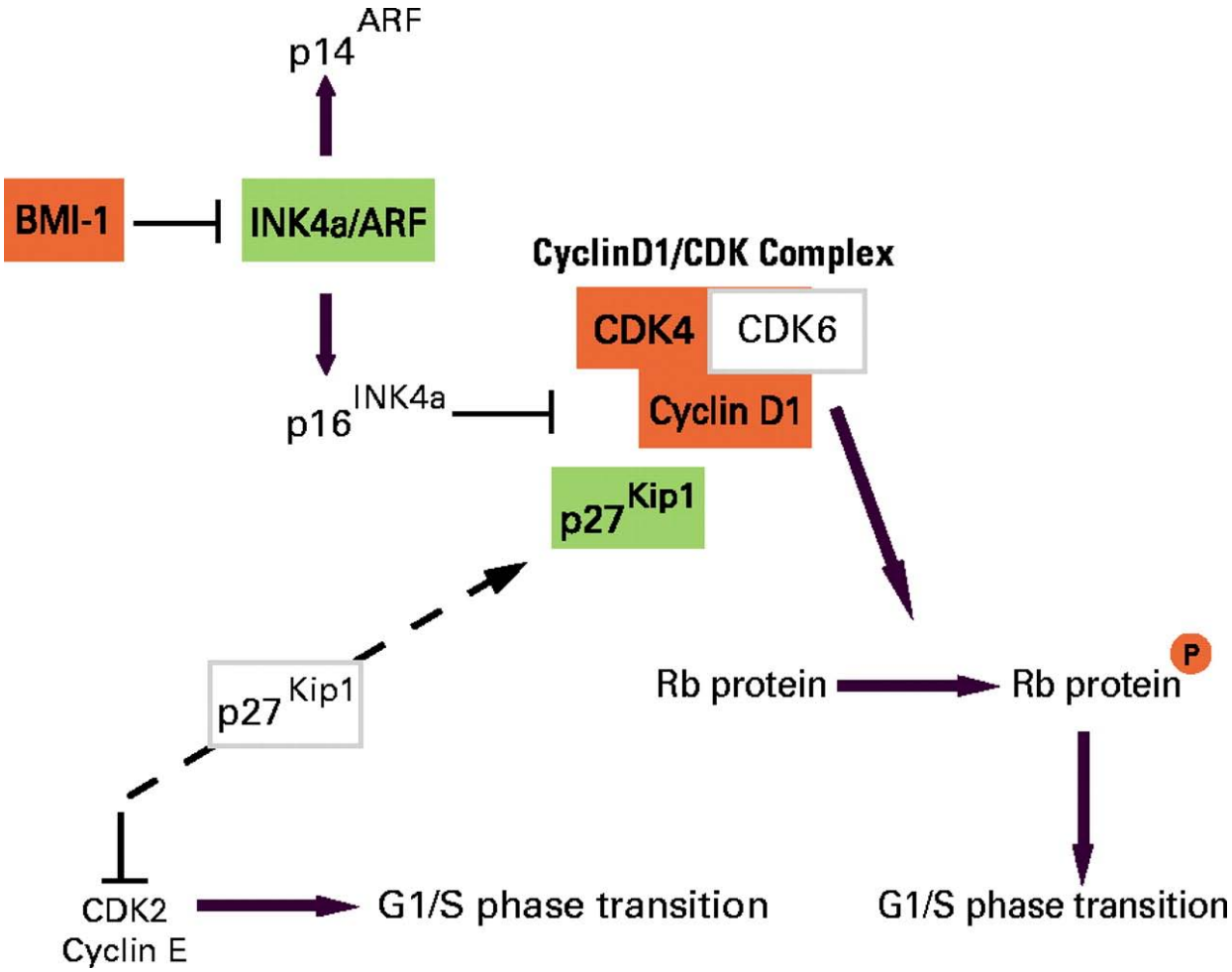


Figure 15. Cell cycle deregulation in mantle-cell lymphoma (MCL).

Molecules with increased expression or function in MCL are colored in yellow and molecules with decreased expression or function are colored in green. Cyclin D1 assembles with cyclin-dependent kinase (CDK) -4 and CDK6 and controls the G1/S-phase transition of the cell cycle. Increased expression of Cyclin D1 in all MCL cases and occasional overexpression of CDK4 promote G1/S-phase transition by phosphorylating the retinoblastoma (Rb) protein. In addition, increased amounts of Cyclin D1 and CDK4 and -6 complexes sequester p27kip1 that is usually bound by Cyclin E/CDK2 complexes, resulting in accelerated cell cycle progression. Homozygous deletion of the INK4a/ARF locus leads to loss of p16 INK4a expression and decreased inhibition of CDK4 and -6. As an alternative to the loss of p16 INK4a, BMI-1 is amplified and/or highly expressed in some MCL cases. [from (Fernandez et al., 2005)]

Significantly, overexpression of *CCND1* alone in transgenic mice is not sufficient to cause the mantle cell lymphoma (Fiancette et al., 2010). It is noteworthy that at least one other gene on 11q13, gluthatione-S-transferase (*GSTπ*), is also highly overexpressed along with *CCND1* after the translocation in MCL (Bennaceur-Griscelli et al., 2004). The over-expression of GSTπ protein with detoxification properties in MCL strongly coincides with resistance to chemotherapy; the effect that serves as one of phenotypic features and main danger of MCL (Thieblemont et al., 2008). Interestingly, despite different chromosomal locations of the *GSTπ* and *CCND1* genes, and the absence of Eμ enhancer near the translocated *GSTπ* allele, they keep coherent expression in MCLs that bear t(11;14)(q13;q32). Thus, transcriptional upregulation cannot be directly explained by translocation of the regulatory elements (HS and Eμ IGH enhancers) on chromosome 14 into relative proximity (120 to 300 kbp) to the *CCND1* gene.

Several treatments are suggested for MCL. There are a number of chemotherapy combinations used to treat MCL. Recent studies show that patients who are treated with chemotherapy plus rituximab (Rituxan®), a monoclonal antibody against CD20 antigen, present on B lymphocytes, show higher initial response rate than with chemotherapy alone (Ying et al., 2012), (Kaplan et al., 2014). Radioimmunotherapy with a monoclonal antibody to enhance its effectiveness (for example Tositumomab/iodine I-131(Bexxar®)) and yttrium 90 ibritumomab tiuxetan (Zevalin®)) are being studied for the treatment of MCL (Skarbnik and Smith, 2012). Bortezomib (Velcade), a proteasome inhibitor with important anticancer activity, has been studied in clinical trials as a single agent and in combination with other agents. It has been approved by the US Food and Drug Administration (US-FDA) for the treatment of patients with relapsed or refractory MCL in 2006 (Koprivnikar and Cheson, 2012). High-dose drug therapy and autologous stem cell transplantation has resulted in high rates of clinical remission

for MCL patients. However, this type of transplant is generally available to patients less than 60 years old (Cheminant et al., 2015).

Most MCL patients will have refractory or recurrent disease. Treatment of recurrent MCL is difficult because of the rapid development of chemotherapy resistance. MCL resistance to conventional therapy could be explained by at least two hallmarks of the disease. First, the classical malignant cells divide slowly, which makes cells less sensitive to chemotherapy. Second, they display a deregulated cell cycle via overexpression of cyclin D1 and inactivation of p53, or via deregulated cellular DNA damage response, mainly through inactivation of ATM (Greiner et al., 2006), which is one of the major safeguards for genome stability (Smith et al., 2010).

Although the probability of cure remains low, there has been notable progress in the treatment of MCL over the last three decades with a near doubling of overall survival. Therefore, new therapies are being developed (for review see (Camara-Clayette et al., 2012)). Among them is epigenetic treatment, a new therapeutic concept which consists of the use of histone deacetylase inhibitors (HDACis) and/or DNA methyltransferase inhibitors (DNMTi).

III – Epigenetic drugs

Epigenetic modifications play an important role in development and disease because of their potential to alter gene expression patterns and key cellular processes (Li, 2002), (Jones and Baylin, 2002). Discovery of a great number of aberrant epigenetic mechanisms in lymphoid malignancies provides a rational basis for the development of drugs targeting epigenetic modifying enzymes (Hassler et al., 2013).

1. DNA methylation inhibitors

The first DNMT inhibiting agents used in the treatment of malignant diseases were analogs of natural nucleosides: 5-azacytidine and 5-aza-2′-deoxycytidine (decitabine) (Cihak, 1974). These two agents have gained US Food and Drug Administration (US-FDA) approval for routine clinical use for myelodysplastic syndrome.

Azacytidine and decitabine, as well as 1-β-D-arabinofuranosyl-5-azacytosine (fazarabine) and dihydro-5-azacytidine (DHAC), belong to variations of deoxycytidine, where all of them are modified at position 5 of the pyrimidine ring – the feature responsible for inhibiting DNMT. On the molecular level, they incorporate into DNA and/or RNA with formation of covalent

complexes with DNMTs in actively proliferating cells. This prevents the DNA from methylation propagation during replication.

However, azacytidine and decitabine are chemically unstable in water and suppress the blood cells growth and proliferation, leading to toxicity problems when used at high levels (Kantarjian et al., 2003). By contrast, at non-cytotoxic concentrations they show favorable demethylation activity for tumor suppressor genes (Bender et al., 1998). 5-azacytidine and decitabine have been tested in hematological malignancies such as chronic and acute myeloid leukemia (CML and AML) and showed promising clinical trials (Issa et al., 2004), (Scandura et al., 2011).

New nucleoside analogs, clofarabine and nelarabine, have been approved for treatment of refractory patients with lymphoblastic lymphoma (LBL) and acute lymphoblastic leukemia (ALL), and cladribine, fludarabine and pentostatin - for the treatment of lymphoid malignancies (for review see (Robak, 2011)).

In vitro studies showed that DNMT inhibitors induce re-expression of methylated tumor suppressor genes, such as p16 and SHP1 in *ALK*-positive anaplastic large cell lymphoma (ALCL) (Hassler et al., 2012), (Han et al., 2006), *CDH13* in DLBCL (Ogama et al., 2004), Lamin A/C in a B-cell lymphoma cell line (Agrelo et al., 2005).

Interestingly, the combinatorial use of DNMT and HDAC inhibitors recently started to gain more and more popularity, showing superior therapeutic outcomes (Thurn et al., 2011), (Cameron et al., 1999), (Steele et al., 2009).

2. Histone deacetylase inhibitors

Histone deacetylase (HDACs) inhibitors are a relatively new class of anti-cancer agents with epigenetic mechanism of action. Due to aberrant activity of HDACs in various types of malignancies, a wide range of HDAC inhibitors (HDACis) have gained considerable popularity as anticancer agents (Seo, 2012), (El-Khoury et al., 2014), (Ogura et al., 2014). HDAC inhibitors have also shown activity against MCL cells in preclinical studies and are being evaluated in patients alone or in combination with other drugs (Camara-Clayette et al., 2012), (Kawamata et al., 2007), (Dasmahapatra et al., 2011).

Information on histone modifications and HDAC inhibitors presented in this chapter was summarized and published in a review (Markozashvili et al., 2015), attached in Annex-III.

a. Types of HDACis

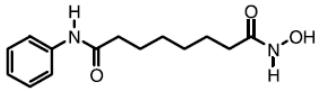
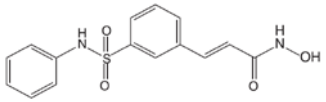
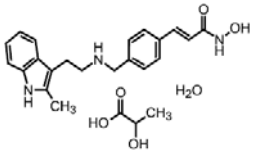
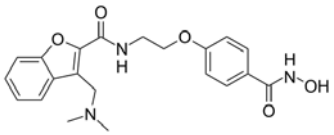
The first generation of HDACis blocks most part of HDACs from class I, while some block class II as well and still fewer also block class IV. Such HDACis are called pan-inhibitors, because of their property of inhibiting several classes. In solid tumors, it is often reported that altered expression of individual HDACs takes place. For example, in gastric cancer HDAC1 and 2 are over-expressed, in breast cancer up-regulated HDACs 6 and 8 are associated with increased invasion (Chun, 2015). These discoveries provoked increased interest for synthesis of class-selective or isoform-selective inhibitors. Some examples of such selective inhibitors are: tubacin, which increases acetylation of tubulin, but not that of histones (Haggarty et al., 2003); PCI-34051 which is specific to HDAC8 and is shown to induce apoptosis in T-cell lymphoma

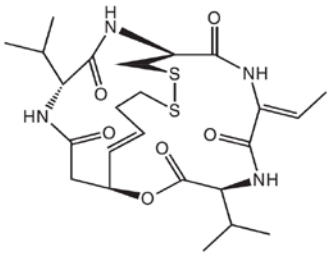
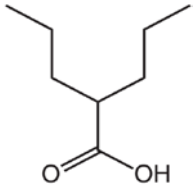
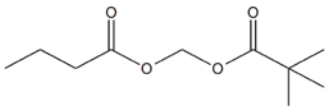
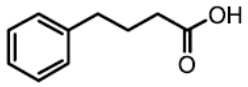
without affecting histone or α-tubulin acetylation (Balasubramanian et al., 2008); and benzamide inhibitor SHI-1:2 which is selective to HDAC1/HDAC2 (Methot et al., 2008). However, complete understanding and characterization of HDAC expression patterns in various cancers is still far. Moreover, in hematological malignancies HDAC expression patterns are not clearly distinguishable (Yang et al., 2015). All this makes it difficult to intelligently target specific HDACs for now.

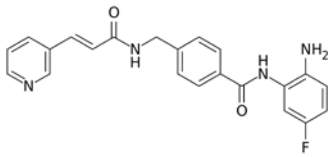
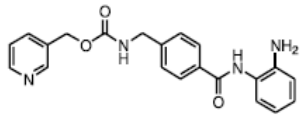
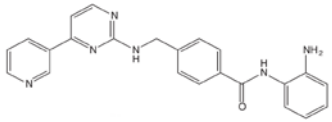
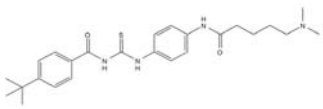
The first inhibitor of HDACs, n-Butyrate was described in the seventies before the actual discovery of histone deacetylases (Riggs et al., 1977). Several other drugs targeting histone acetylation, including Trychostatin A (TSA) and valporoic acid (VPA) were discovered and introduced into clinical practice for treatment of diseases non-connected with cancer. Discovery of the link between histone acetylation and cancer has sparked an interest for HDACi and several new drugs were developed during the last ten years. These drugs can be divided into several chemically distinct groups: aliphatic acids (phenylbutyrate, valporoic acid), benzamides (entinostat), cyclic peptides (romidepsin), and hydroxamates (TSA, vorinostat/SAHA). The HDAC inhibitors that have been tested in lymphoid malignancies are summarized in **Table 4**.

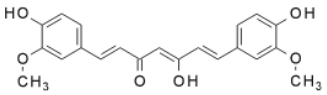
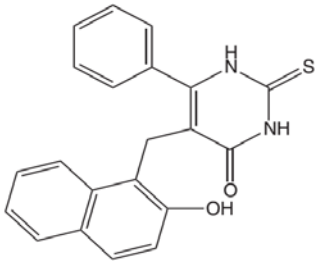
Vorinostat or ***SAHA*** is the first HDACi approved by the United States Food and Drug Administration (U.S. FDA) in 2006 for clinical use in patients with cutaneous T-cell lymphoma (Zolinza®, Merck and Co., Inc.) (Grant et al., 2007); (Bolden et al., 2006). First synthesized in 1998, it was shown to induce erythroid differentiation (Richon et al., 1998). SAHA is pan-HDAC inhibitor that blocks HDAC enzymes from class I, II, and IV. It was also tested in Hodgkin lymphoma, follicular lymphoma, marginal zone lymphoma and mantle cell lymphoma with promising results in relapsed/refractory indolent follicular lymphoma (Kirschbaum et al., 2011); (Ogura et al., 2014). Vorinostat belongs to the hydroxamic acid group.

Table 4. HDAC inhibitors: structure and function.

Drug	HDAC target (potency)	Type of cancer in which tested	Clinical trial phase	Structure	Mechanism of action	Reference
Hydroxamic Acids						
Vorinostat (SAHA)	Class I, II (μM)	Hodgkin's lymphoma, non-Hodgkin's lymphoma and multiple myeloma	Food and Drug Administration approved for CTCL I-II-III		Suppresses genes that promote uncontrolled growth, promotes apoptosis, interrupts cell cycle progression. TD; GA; AI; AE; MF; AU; S; PP; ROS-CD	(Dokmanovic et al., 2007) (Eot-Houllier et al., 2009)
Belinostat (PXD-101)	Class I, II	B-cell lymphoma, MCL, HL, PTCL and CTCL	FDA approval for Refractory or Relapsed Peripheral T cell lymphoma		GA; A	(Gimsing, 2009) (De Souza and Chatterji, 2015) (Foss et al., 2015) (Zain and O'Connor, 2010)
Panobinostat (LBH-589)	Class I and II (nM)	CTCL, PTCL, myelogenous leukaemia	I-II-III		GA; A; ROS-CD	(Dokmanovic et al., 2007) (Eot-Houllier et al., 2009)
Abexinostat (PCI-24781, CRA-024781)	Class I and II (nM)	B-cell lymphoma	II		Promotes apoptosis, interrupts cell cycle progression	(Rivera-Del Valle et al., 2010) (Eot-Houllier et al., 2009) (Morschhauser et al., 2015)

Cyclic Peptides						
Romidepsin (FK228)	Class I and II (nM)	CML, AML, MDS, multiple myeloma, CTCL and PTCL	FDA approval for CTCL treatment		Suppresses genes that promote uncontrolled growth, promotes apoptosis, interrupts cell cycle progression. TD; GA; A; AI; AE; MF; ROS-CD	((Nakajima et al., 1998) (Byrd et al., 2005) (Piekarz et al., 2009) (Klimek et al., 2008) (Bates et al., 2010) (Grant et al., 2010) (Dokmanovic et al., 2007)
Aliphatic Acids						
Valproic Acid	Class I and IIa (mM)	Myelodysplastic syndromes Lymphocytic leukaemia	I-II		Induces oxidative stress. TD; GA; A; S	(Dokmanovic et al., 2007) (Cotto et al., 2010) (Eot-Houllier et al., 2009)
Pivanex (AN-9)	µM	T-ALL Chronic myelocytic leukemia	I - II		TD; GA; A	(Dokmanovic et al., 2007) (Eot-Houllier et al., 2009) (Batova et al., 2002)
Sodium phenyl butyrate (NaPB)	Class I and IIa (mM)	Myelodysplastic syndromes	I		TD; GA; A; AI; AE	(Dokmanovic et al., 2007) (Cortez and Jones, 2008) (Eot-Houllier et al., 2009)

Benzamides						
Chidamide (CS055)	Class I and IIb (nM)	PTCL	Chinese FDA approval for peripheral T-cell lymphoma			(Gong et al., 2012)
Entinostat (MS-275)	Class I (µM)	Lymphoid malignancies	I-II		TD; GA; A; AI; AE; ROS-CD	(Dokmanovic et al., 2007) (Eot-Houllier et al., 2009) (Cortez and Jones, 2008)
Mocetinostat (MGCD0103)	Class I, IV (µM)	Hodgkin's disease, advanced leukemia	II		TD; GA; A	(Cotto et al., 2010) (Dokmanovic et al., 2007) (Eot-Houllier et al., 2009) (Garcia-Manero et al., 2008)
Tenovin-6	SIRT1 and SIRT2	CML, ALL	preclinical		Induces p53 acetylation, inhibits tumor growth, and eliminates cancer stem cells in combination with Imatinib	(Li et al., 2012) (Seto and Yoshida, 2014) (Jin et al., 2015)

Other						
Curcumin, Diferuloyl-methane	Class I	Hodgkin's lymphoma, non-Hodgkin's lymphoma	preclinical		Inhibits DNMT activity by covalently blocking the catalytic thiolate of C1226 of DNMT1. Induces apoptosis in cancer cells, has anti-inflammatory and anti-oxidant properties	(Liu et al., 2009) (Eot-Houllier et al., 2009)
Cambinol	SIRT1 and SIRT2	BL	preclinical		Increases p53 acetylation	(Heltweg et al., 2006) (Seto and Yoshida, 2014)

GA, growth arrest; TD, terminal differentiation; A, apoptosis; AI, cell death by activating intrinsic apoptotic pathway; AE, cell death by activating extrinsic apoptotic pathway; MF, mitotic failure; AU, autophagic cell death; S, senescence; PP, polyploidy; ROS-CD, reactive oxygen species – facilitated cell death. CTCL - of cutaneous T-cell lymphoma. PTCL - peripheral T-cell lymphoma. BL - Burkitt lymphoma. CML - chronic myelogenous leukemia. MDS - Myelodysplastic syndrome. MCL – Mantle cell lymphoma. ALL - acute lymphoblastic leukemia.

Romidepsin (FK228) is a cyclic peptide isolated from *Chromobacterium violaceum* (Nakajima et al., 1998). It has been investigated in patients with hematological malignancies such as CML, AML, MDS, multiple myeloma, cutaneous T-cell lymphoma (CTCL) and peripheral T-cell lymphoma (PTCL) (Byrd et al., 2005); (Klimek et al., 2008). The former has showed promising results (Piekarz et al., 2009). In 2009, it was granted U.S. FDA approval for CTCL treatment, and for PTCL in 2011 (Istodax®, Gloucester Pharmaceuticals - a subsidiary of Celgene Corp). A good correlation between drug exposure level and histone acetylation has been revealed (Bates et al., 2010).

Belinostat (PXD-101), another hydroxamic acid derivative HDAC inhibitor, has shown clinical response in patients with B-cell lymphoma (Gimsing, 2009), mantle cell lymphoma and HL (Zain and O'Connor, 2010), PTCL and CTCL (Foss et al., 2015). It is the third drug which received the U.S. FDA approval in 2014 for the treatment of refractory or relapsed PTCL (Beleodaq®, Spectrum Pharms, Inc.) (De Souza and Chatterji, 2015).

Chidamide (CS055) is a new benzamide type of HDAC inhibitor with subtype selective activity against HDAC1, 2, 3 and 10. Chidamide is being studied in multiple clinical trials as a single agent or in combination with chemotherapeutic agents for the treatment of various hematological and solid cancers (Gong et al., 2012); (Dong et al., 2012). It induces growth arrest and apoptosis in blood and lymphoid-derived tumor cells, activates NK- and CD8 T-cell-mediated antitumor activity, stimulates differentiation of tumor stem cells and represses genes associated with drug resistance (Gong et al., 2012). In 2015, chidamide obtained approval (Epidaza®) from the Chinese FDA for the treatment of relapsed or refractory PTCL.

Givinostat is a hydroxamic pan HDAC inhibitor recently tested in several hematological malignancies such as multiple myeloma, HL and myeloproliferative disease. Clinical efficacy

has been reported in patients with Polycythemia Vera and Essential Thrombocythemia (Rambaldi et al., 2010).

Panobinostat is also a hydroxamic acid analog with pan HDAC inhibitor activity. It has been studied in various hematological malignancies, revealing good clinical results in patients with CTCL (Duvic et al., 2013), HL (Dickinson et al., 2009), AML (DeAngelo et al., 2013). Panobinostat has been shown to induce autophagy, thus combination of that drug with autophagy inhibitor can increase its antitumor effects (Rao et al., 2012).

Valproic Acid is a member of the short chain fatty acids and an inhibitor of HDACs class I. VPA is a potent and very selective inhibitor of STAT3 (Lee and Kim, 2012). In preclinical studies on chronic lymphocytic leukaemia (CLL), it showed cell-killing activity by triggering apoptotic pathways (Bokelmann and Mahlknecht, 2008). VPA is undergoing evaluation in India as therapy for CLL (Ganesan et al., 2009).

Entinostat, a benzamide derivative class member specific to class I HDACs, has been tested in several lymphomas (Saito et al., 1999). It is tolerated well, both alone and in combination with other drugs (Pili et al., 2012).

Mocetinostat is another synthetic benzamide with an inhibiting specificity for HDACs class I and IV. It has a prolonged pharmacodynamic effect (Bonfils et al., 2008). This drug was evaluated for the clinical efficacy in patients with follicular lymphoma (Crump et al., 2008), Hodgkin lymphoma (Bociek et al., 2008) and AML (Siu et al., 2008).

Abexinostat, or ***S78454***, is a novel hydroxamic acid pan HDAC inhibitor. Abexinostat induces apoptosis and histone alterations in a caspase-8 depended manner in acute leukemia cells ((Rivera-Del Valle et al., 2010)). In clinical studies Abexinostat showed promising durable

responses in follicular lymphoma, B-cell lymphoma, chronic lymphocytic leukaemia patients and is currently undergoing phase II of clinical trials ((Morschhauser et al., 2015)).

Cambinol, a sirtuin inhibitor, has been reported to have antitumor activity in Burkitt's lymphoma by increasing p53 acetylation (Heltweg et al., 2006).

b. Mechanisms of HDACis action

HDACis have a variety of biological effects across multiple pathways, including selective alterations of gene expression and post-translational regulation of pro- and anti-apoptotic genes from «extrinsic» (death-receptor) and «intrinsic» (mitochondrial) apoptosis pathways, angiogenesis inhibition, generation of reactive oxygen species (ROS), autophagy, regulation of DNA damage and repair, and other (Kim and Bae, 2011), (Chun, 2015), (Xu et al., 2007). However, the mechanism of HDACis action by which the clinical activity is mediated is not completely discovered and remains obscure till date.

One of the reasons which makes the treatment with HDAC inhibitors favorable is that normal cells are relatively resistant to the treatment, whereas cancer cells are more responsive and undergo growth arrest, inhibited differentiation and apoptosis. Until now, there is no clear explanation of HDACis selectivity for cancer *vs.* normal cells. One of the possible mechanisms may induce DNA damage, which only normal but not malignant cells can repair (Lee et al., 2010).

The effect of HDACi on lymphoid malignancies can be improved by combination with other drugs, e.g. DNA damaging agents such as topoisomerase II poisons or DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (Decitabine) (Thurn et al., 2011). Tenovin-6, sirtuin inhibitor,

together with BCR-ABL tyrosine kinase inhibitor (Imatinib) was shown to be effective in chronic myelogenous leukemia (Li et al., 2012). A combination of new potent HDAC inhibitors, including abexinostat, belinostat, panobinostat, entinostat and vorinostat decitabine may lead to an increase in clinical benefits.

c. HDACi and epigenetic regulation in lymphoid malignancies

Lymphoid malignancies are provoked by translocations (for review see (Nussenzweig and Nussenzweig, 2010)). Some of these translocations result in gene fusions. Most gene fusions involve transcription factors or chromatin regulators. In myeloid leukemias, the fusion proteins such as PML-RAR and AML1-ETO form stable complexes with HDACs (Grignani et al., 1998a); (Minucci et al., 2000); (Lin and Evans, 2000). This provided a rational basis for the first study of HDACi in leukemia.

Treatment of mice having PML-RAR induced leukemia with valporoic acid increased survival and induced apoptosis of leukemic cells (Insinga et al., 2005). This study has prompted other researchers to test HDACi on other lymphomas and leukemias, although the mechanisms of these lymphomas are different from myeloid leukemias. Indeed, in B-cell lymphomas, juxtaposition of gene regions may lead to over-expression of oncogenes such as *CCND1*, *CMYC* (Dalla-Favera et al., 1982); (Taub et al., 1982) or *BCL2* (Bakhshi et al., 1985); (Cleary and Sklar, 1985).

Recently, it has been shown that translocations also lead to large-scale epigenetic changes (Liu et al., 2004), large-scale reorganisation of nuclear organization and aberrant gene expression (Harewood et al., 2010); (Allinne et al., 2014). At the same time, most documented

effects of HDACi relate to apoptosis associated with increased expression of pro-apoptotic genes and decreased expression of anti-apoptotic genes, leading to cell death via one of the following pathways: inhibition of angiogenesis, generation of reactive oxygen species, autophagy or apoptosis. Currently our understanding of the immediate and prolonged action of HDACi on cells is insufficient and further studies in this direction are required to make full advantage of these powerful anticancer drugs.

IV - Conclusions and objectives

Mantle cell lymphoma (MCL) is a rare and very aggressive from of non-Hodgkin's lymphoma (Fisher et al., 1995). Involvement of epigenetic mechanisms in establishment and maintenance of MCL was argued in the recent years. The translocation event that occurs in pre-B lymphocytes during erroneous recombination of IgH gene juxtaposes the cyclin D1 (*CCND1*) locus on chromosome 11 with the immunoglobulin heavy chain (IgH) locus on chromosome 14. As a result, *CCND1*, which is not expressed in quiescent normal lymphoid cells, becomes active (Jaffe et al., 2001). Intriguingly, coordinated overexpression of glutathione S transferase pi gene (*GSTπ*) on der11 together with *CCND1* gene, despite their different chromosomal location, has been found in MCL cells (Bennaceur-Griscelli et al., 2004). This transcriptional upregulation cannot be directly explained by translocation of the regulatory elements (HS and Eμ IGH enhancers) on chromosome 14 into the relative proximity (120 to 300 kbp) of the *CCND1* gene.

There has been notable progress in the treatment of MCL over the last three decades with a near doubling of overall survival, even though the probability of cure remained low. Therefore new therapies are being developed (for review see (Camara-Clayette et al., 2012)). Among them is epigenetic treatment, a new therapeutic concept which consists of the use of histone deacetylase inhibitors (HDACis) and/or DNA methyltransferase inhibitors (DNMTis).

Histone deacetylase inhibitors affect histone deacetylases (HDACs) which are directly involved in the alteration of the structural components of chromatin and other non-histone proteins (Walkinshaw and Yang, 2008). Thus, HDACis can influence gene expression patterns and regulate different cell processes (Gray et al., 2004), (West and Johnstone, 2014). In MCL cell lines, HDACs inhibitors were shown to have antiproliferative effects, and paradoxically they decreased the cyclin D1 protein level in the cells (Heider et al., 2006). Until now, there is no clear understanding of HDACis mechanism of action and explanation of such an effect on cyclin D1 in MCL. Therefore, a study of «epigenetic landscape» in 11q13 and 14q32 loci should significantly advance our knowledge about the mechanisms of expression of *GSTπ* and *CCND1* in MCL.

We believe that structural properties of higher-order chromatin conformation may significantly change transcription of a gene group if they are localized in the same or different chromatin loops (Vassetzky et al., 2000). We have previously shown that the translocated regions relocalize in the intranuclear space, and that change of surroundings might be a complementary epigenetic mechanism for gene activation or repression in MCL (Allinne et al., 2014). Such a relocalization might be accompanied by epigenetic changes in the translocated regions; this is an additional argument for the use of epigenetic drugs in MCL.

The purpose of the present work was to study chromatin structure in the rearranged (11;14)(q13;q32) locus in MCL cells as compared to the 11q13 and 14q32 loci in normal human lymphocytes. We then studied the effect of different HDACis on the rearranged (11;14)(q13;q32) locus at several levels: histone acetylation / methylation, chromatin state and gene expression.

We have shown that t(11:14)(q13;q32) translocation leads to overexpression of not only *CCND1* but a big group of genes spanning over 15 Mb around the translocated region. The same genes, sensitive to deregulation by t(11;14) translocation, react to the HDACi treatment by increasing their expression. Importantly, while abexinostat stimulates genome-wide desegregation of heterochromatin, genes' promoters stay shielded from its effect. It has been previously shown that HDACi paradoxically decrease Cyclin D1 protein amount (Heider et al., 2006). We have demonstrated that it happens not via direct influence on *CCND1* gene expression. Thus, the data supports the idea of an indirect mechanism of HDACi abexinostat action.

RESULTS

1 - Epigenetic state of rearranged loci in MCL and control cell lines

The first part of the work aimed to analyze the chromatin state and gene expression levels in the 11q13 locus that is translocated in MCL cells in order to dissect the nature of *CCND1* gene upregulation in MCL.

As discussed above, translocation t(11;14) leads to cyclin D1 gene (*CCND1*) activation. Initially, a direct influence of a strong *IgH* enhancer was hypothesized to upregulate *CCND1* transcription. However, cyclin D1 gene may be located at a long distance from the chromosome breakpoint (200 kb), which makes direct enhancer-promoter effect much less probable. Moreover, gluthatione-S-transferase (*GSTP1*) located on the derivate of 11 chromosome as well reveals overexpression after the translocation in MCL (Bennaceur-Griscelli et al., 2004). Previously, we have shown that 11q13 locus relocalizes from the nuclear periphery towards the transcriptionally active nuclear center and nucleolus (Allinne et al., 2014). This may lead to activation of the entire locus. All these data suggest an epigenetic mechanism of gene upregulation in MCL, rather than simple enhancer-promoter effect. Therefore, we wanted to analyze the chromatin structure and gene expression in the rearranged loci in order to assess its epigenetic state.

1. *Activated epigenetic landscape in the 11q13 and 14q32 loci after the translocation (11;14) in MCL cells.*

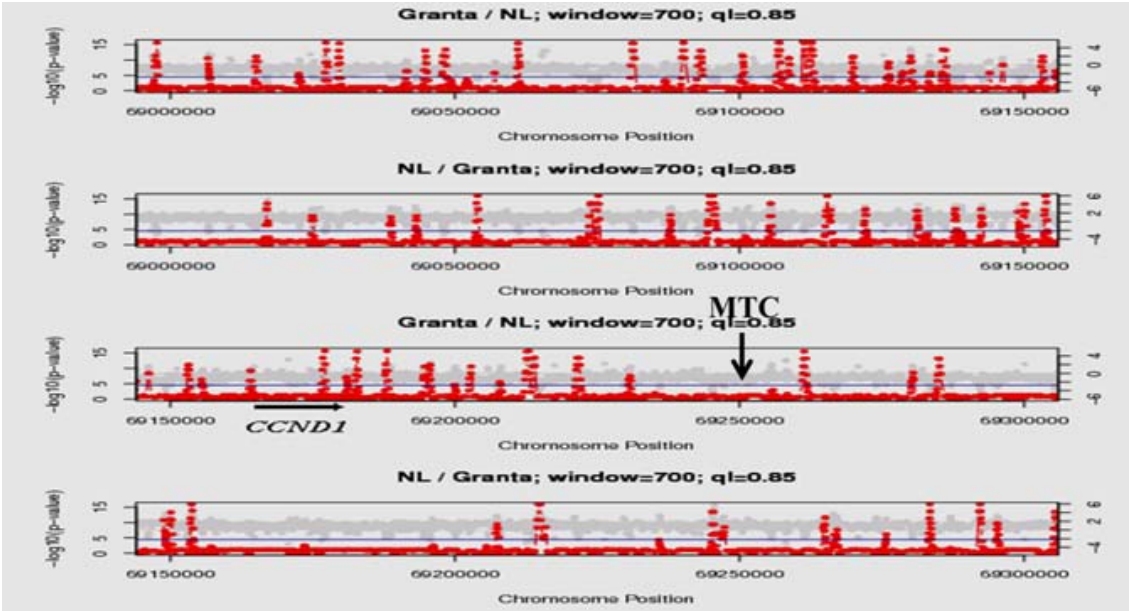
Large-scale movements of chromatin after the translocation (11;14)(q13;q32) in MCL may provoke global changes in histone modifications of chromatin in the translocated loci. To study this, we have isolated nucleosomes containing histone H3 acetylated at lysine 9 (H3K9Ac; mark of active chromatin) and di-methylated at the same position (H3K9me2; mark of facultative heterochromatin) from normal human B-lymphocytes (NBL) and MCL Granta-519 cell line using specific antibodies. DNA was extracted from the samples and used as a probe for hybridization with a custom NimbleGene genomic microarray covering a 1.4 Mb region in 11q13 locus (including *CCND1* and *GSTP1* genes) and 2 Mb region in 14q32 locus (including *IGH* gene). The microarray was prepared with a median probe spacing of 70 bp.

The total DNA samples were used for reference hybridization in both experimental sets. The results of hybridization with microarrays were analyzed using the ACME (Algorithm for Capturing Microarray Enrichment) program (Scacheri et al., 2006a), (Scacheri et al., 2006b) as described in Materials and Methods. The program identifies the probes representing the genomic region under study and scans this region using a window size which can be user-defined. We have chosen a window size of 400 bp because it will cover DNA fragments containing up to two nucleosomes. The p-values assessing a possible association of DNA with either H3K9Ac or H3K9me2 were calculated and represented as graphs along the chromosomal regions studied. The distributions of H3K9Ac and H3K9me2 peaks in Granta-519 were referred to that in NBL and vice versa allowing detection of peaks which are presented in MCL, but not in the control.

Results obtained for the two selected regions on chromosomes 11 and 14 are shown in **Figure 16.** Alignment with gene positions, corresponding to assembly GH18, is shown in the lower part of the figures. From the analysis of the data presented in **Figure 16A**, it is clear that the 1.4 Mb segment in the 11q13 locus, which contains the *CCND1* and *GSTP* genes, had more acetylation peaks in Granta-519 (190 Ac peaks in Granta-519 vs. 113 Ac peaks in NBL). Three distinct H3K9Ac peaks were observed around *CCND1* gene. From the analysis of the 14q32 locus reported in **Figure 16B**, peaks corresponding to H3K9Ac were found scattered all over the region, although there were more active chromatin marks in MCL as compared to NBL.

This more active chromatin state in the loci after indicates rather an epigenetic mechanism of gene deregulation after the translocation than mere gene-enhancer effect.

A



B

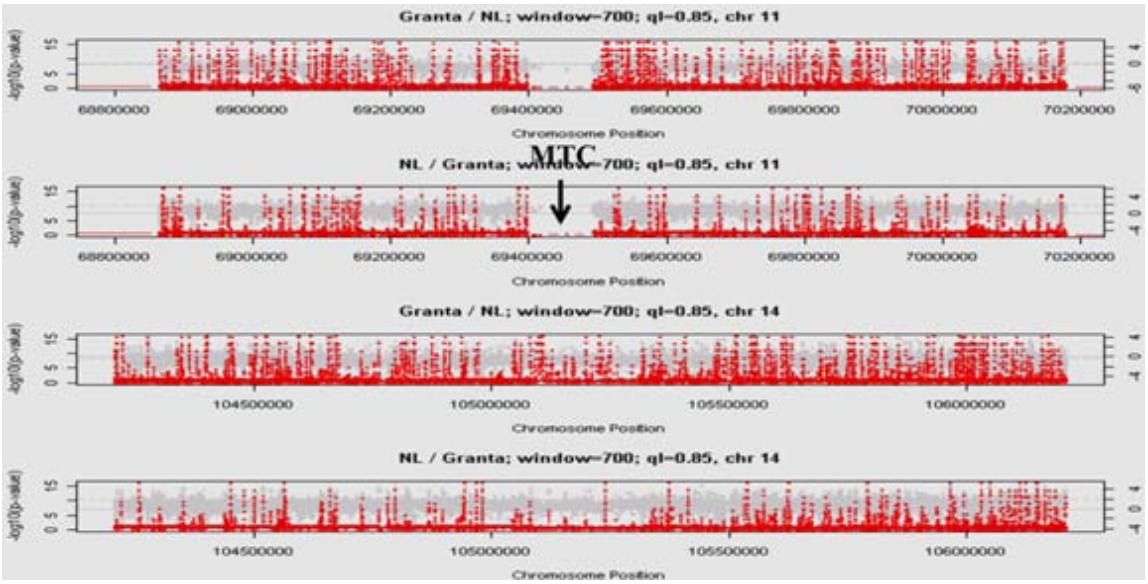


Figure 16. Distribution of H3K9Ac chromatin marks within the translocated loci.

X-axis: log_2-ratio Granta-519 / NBL (1st and 3rd lines) and NBL / Granta-519 (2nd and 4th lines). The blue lines show the significance threshold higher than the False Discovery Rates (FDR); MTC, the Major Translocation Cluster in MCL. Gene coordinated are given in correspondence with HG18 assembly. (**A**) A zoom on 300 Kb segment in 11q13 locus. The horizontal arrow indicates *CCND1* gene. (**B**) Entire segments analyzed: 1.4Mb in 11q13 locus (1st and 2nd lines) and 3Mb in 14q32 locus (3rd and 4th lines).

2. *Analysis of gene expression in the translocated 11q13 region in MCL cells reveals upregulated genes adjacent to the translocation point on chromosome 11.*

We have first wanted to determine whether the t(11;14) perturbs expression of genes other than *CCND1* in the vicinity of the translocation point. In order to identify this, we have collected data from Gene Expression Omnibus (GEO) database, corresponding to different microarray platforms, and compared gene expression in MCL to normal naïve B cells.

After downloading the raw expression data, we have normalized it and found differentially expressed genes in the 15 Mb 11q13 locus adjacent to the translocation point. A procedure using percentile of the distribution of coefficient variability values was used to filter out the least variable probe sets. The threshold for this filtering was set at 0.5. The differentially expressed genes between MCL and NB lymphocytes were identified using limma R package from Bioconductor with the Benjamini-Hochberg procedure for multiple test adjustment. The adjusted p-value threshold was set to 0.05. We have identified 334 genes, 36 of them being activated after the t(11;14) translocation (latter referred as *upregulated* genes) and 298 genes which do not change their expression after the translocation (latter referred as *not-upregulated* genes) (

Table 5).

Table 5. 36 upregulated genes spanned over 15 Mb region near the (11;14) translocation point.

Gene expression data was extracted from GEO database and analyzed as described in Materials and Methods. Expression rate represents the ratio of a gene expression in MCL relative to control.
Start point of a gene is shown according to the UCSC genome browser (assembly HG19).
Bold – ten genes chosen for further analysis. Underlined – genes around the translocation point.

Start point	Gene	Expression rate	Gene name
60739115	***CD6***	**1.80**	**CD6 molecule**
60869930	***CD5***	**1.94**	**CD5 molecule**
61100654	*DAK*	1.44	Dihydroxyacetone kinase 2 homolog (S. cerevisiae)
62201016	*AHNAK*	4.54	AHNAK nucleoprotein
62360675	***MTA2***	**1.43**	**Metastasis associated 1 family, member 2**
62457747	*BSCL2*	1.71	Bernardinelli-Seip congenital lipodystrophy 2 (seipin)
62623518	*SLC3A2*	1.75	Solute carrier family 3
63341934	*PLA2G16*	2.36	Phospholipase A2, group XVI
63448922	*RTN3*	1.92	Reticulon 3
64037300	***BAD***	**1.52**	**BCL2-associated agonist of cell death**
64494383	*RASGRP2*	1.54	RAS guanyl releasing protein 2 (calcium and DAG-egulated)
64513861	*PYGM*	1.80	Phosphorylase, glycogen, muscle
64692180	*PPP2R5B*	1.37	Protein phosphatase 2, regulatory subunit B', beta isoform
64794910	*SNX15*	1.47	Sorting nexin 15
65337943	*SSSCA1*	1.41	Sjogren syndrome/scleroderma autoantigen 1
65479489	***KAT5***	**1.39**	**K(lysine) acetyltransferase 5**
65686728	*DRAP1*	1.71	DR1-associated protein 1 (negative cofactor 2 alpha)
66059373	*TMEM151A*	1.51	Transmembrane protein 151A
66278119	*BBS1*	1.46	Bardet-Biedl syndrome 1
66330935	***CTSF***	**1.41**	**Cathepsin F**
66610883	*RCE1*	1.44	RCE1 homolog, prenyl protein peptidase (S. cerevisiae)
67195935	*RPS6KB2*	1.32	Ribosomal protein S6 kinase, 70kDa, polypeptide 2
67205518	*CORO1B*	2.44	Coronin, actin binding protein, 1B
67259239	*PITPNM1*	1.32	Phosphatidylinositol transfer protein, membrane-associated
67351066	***GSTP1***	**2.92**	**Glutathione S-transferase pi 1**
67820326	*CHKA*	1.60	Choline kinase alpha
68080108	*LRP5*	1.46	Low density lipoprotein receptor-related protein 5
68522088	*CPT1A*	2.56	Carnitine palmitoyltransferase 1A (liver)
68671319	*IGHMBP2*	1.52	Immunoglobulin mu binding protein 2
69455873	***CCND1***	**25.14**	**Cyclin D1**
69480331	***ORAOV1***	**1.42**	**Oral cancer overexpressed 1**
70244612	*CTTN*	1.41	Cortactin
71145457	*DHCR7*	1.43	7-dehydrocholesterol reductase
71639768	*RNF121*	1.31	Ring finger protein 121
71900602	*FOLR1*	2.34	Folate receptor 1 (adult)
75526212	***UVRAG***	**1.58**	**UV radiation resistance associated gene**

3. *Manuscript "Histone deacetylase inhibitor abexinostat affects chromatin organization and gene transcription in normal B cells and in mantle cell lymphoma"*

Histone deacetylase inhibitors, drugs of new generation recently applied in anticancer therapy (Richon and O'Brien, 2002) are now used in therapy of lymphoid malignancies (Ribrag, 2015). These drugs relate on histone deacetylases (HDACs) which are directly involved in alteration of the structural components of chromatin (Walkinshaw and Yang, 2008), (Tan et al., 2010). In MCL cell lines, HDACs inhibitors (HDACis) were shown to have anti-proliferative effects, and paradoxically they downregulate cyclin D1 levels (Heider et al., 2006), (Alao et al., 2004).

Here, we wanted to evaluate HDACis effect on chromatin structure and 11q13 genes' expression before and after the translocation t(11;14). The HDACi abexinostat (lab. Servier) has been chosen for this purpose.

Histone deacetylase inhibitor abexinostat affects chromatin organization and gene transcription in normal B cells and in mantle cell lymphoma

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SUMMARY

Mantle cell lymphoma (MCL) is a rare lymphoma caused by the t(11:14) juxtaposing the cyclin D1 (*CCND1*) locus on chromosome 11 and the immunoglobulin heavy chain (*IgH*) locus on chromosome 14. Several new treatments are proposed for MCL, including histone deacetylase (HDAC) inhibitors. In the present paper we show that a large amount of genes is overexpressed in the translocation region on chromosome 11 and show that most part of these genes are sheltered from the direct effect of HDAC inhibitor abexinostat (S78454, PCI-24781).

INTRODUCTION

Mantle cell lymphoma (MCL) is a rare disease accounting for 5–7% of non-Hodgkin lymphomas in adults [1]. It directly linked to the t(11;14)(q13;q32) juxtaposing the cyclin D1 (*CCND1*) locus on chromosome 11 and the immunoglobulin heavy chain (*IgH*) locus on chromosome 14 in early B-cells. As a result, *CCND1* which is not expressed in quiescent normal lymphoid cells, becomes active [2], although there exists a small number of cases that express cyclin D2 or D3 instead of *CCND1* [3]. In addition to *cyclin D1* overexpression, supplementary genetic alterations appear to disturb the cell cycle machinery and interfere with the cellular response to DNA damage. MCL resistance to conventional therapy could be explained by at least two hallmarks of the disease: first the classical malignant cells are slow dividing and second they display a deregulated cell cycle, via overexpression of *cyclin D1* and inactivation of P53 or deregulated cellular DNA damage response mainly through inactivation of ATM [4], which is one of the major safeguards for genome stability [5].

Significantly, overexpression of *CCND1* alone in transgenic mice is not sufficient to cause the mantle cell lymphoma [6]. It is noteworthy that at least one other gene on 11q13, gluthatione-S-transferase (*GSTp*), is also highly overexpressed along with *CCND1* after the translocation in MCL [7]. This transcriptional upregulation cannot be directly explained by translocation of the

regulatory elements (epsilon and mu IGH enhancers) on chromosome 14 into the relative proximity (120 to 300 kbp) to the *CCND1* gene. Indeed, we have recently shown that t(11;14) led to relocalization of the *CCND1* locus in the nuclear space to the perinucleolar region where it was regulated by an abundant nucleolar protein nucleolin [8]. Reorganization of the nuclear space is also observed in other cancers [9, 10]. Chromosomes in the eukaryotic nuclei are arranged in chromosomal territories that occupy a specific place in the nuclei (for review see [11]. Gene-poor chromosomes are localized to the nuclear periphery, whereas gene-rich chromosomes have a more central position in the nucleus. Genes and gene domains may relocalize within the territories upon their activation or repression [12, 13] and also when damaged reviewed in [14].

Although the prognosis has clearly improved for MCL over the last few decades, the probability of cure remained low, therefore new therapies are being developed (for review see [15]). Histone deacetylase (HDAC) inhibitors are one class of drugs that are currently used in clinical trials [15]. Histone deacetylase inhibitors represent epigenetic agents that modify chromatin structure through histone acetylation [16]. HDAC inhibitors cause cell death through multiple mechanisms, including upregulation of death receptors, induction of oxidative injury, and disruption of DNA repair [17]. HDAC inhibitors have also shown activity against MCL cells in preclinical studies and are being evaluated in patients alone or in combination with other drugs [15, 18, 19], reviewed in [20].

Abexinostat is a new broad-spectrum phenyl hydroxamic acid HDAC inhibitor currently being evaluated in phase I-II clinical trials. It has shown signs of clinical activity in Phase I in relapsed and refractory lymphoma [21]. In lymphoma cell lines, abexinostat, at clinically achievable concentrations, induced concentration-dependent apoptosis which was dependent on caspase and ROS production [22]. In the present work we have evaluated the effect of abexinostat on cancer cells and found out that it alters transcription and epigenetic signature in only a small subset of genes, regardless of global changes in the nuclear architecture triggered by the treatment.

MATERIALS AND METHODS

Cell cultures

The human Mantle Cell Lymphoma (MCL) cell lines Granta-519, Jeko-1, UPN-1, Mino and NCEB-1 were used in experiments. Lymphoblastoid cell lines RPMI-8866, Priess, Remb1, IARC-211 and IARC-171 were used as controls.

RPMI-8866, Priess, Remb1, IARC-211, IARC-171, Granta-519 and Mino cells were maintained in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 1% penicillin-streptomycin (Invitrogen). UPN-1 and NCEB-1 cells were cultured in MEM alpha medium (Invitrogen, Cergy Pontoise, France) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Perbio science), 2 mM L-glutamine, and 1% penicillin-streptomycin (Invitrogen). Jeko-1 cells were maintained in RPMI 1640 supplemented with 20% fetal bovine serum, 2 mM L-glutamine, and 1% penicillin-streptomycin (Invitrogen). Cells were cultured at 37°C in a humidified 5% CO2 atmosphere.

Abexinostat treatment

Stock solution of abexinostat in dimethylsulfoxide (DMSO) at 0.1 mM was conserved at -20°C. Cells were incubated in the appropriate growth media supplemented with 100 nM abexinostat or DMSO as the control during 1 and 24 hours at normal growth conditions.

3D-fluorescence immunodetection

Cells were immobilized on glass coverslips coated with Poly-D-lysine hydrobromide (Sigma). The cells were then treated as previously described to preserve their three-dimensional (3D) structure [23]. Heterochromatin clusters were immunodetected using rabbit anti-H3K9me3 (Upstate) antibodies and goat anti-rabbit Alexa 633 (Invitrogen) antibody. DNA was counterstained with 4,6 diamidino-2-phenylindole (Vectashield, Vector) or Bobo1 (Invitrogen).

Confocal microscopy, image processing, and statistical analysis were carried out as described [8].

Chromatin immunoprecipitation (ChIP)

For chromatin isolation, cells were fixed with 1% ammonium persulfate and sonicated in a lysis buffer (50mM Tris-HCl, pH 8.0, 10mM EDTA, 1% SDS, 0.2mM PMSF, 1% PIC) with 10 cycles of 20 sec pulse-on, 30 sec pulse-off, 40% amplification. The non-solubilized material was removed by centrifugation at 16,000g for 10 min. The size of chromatin fragments (1-3 nucleosomes) was monitored by electrophoresis in a 1% agarose gel after rev-crosslinking and treatment with 5 µg/ml RNaseA and 2 µg/ml proteinase K.

Chromatin immunoprecipitation was performed as following: 21 µg of chromatin solution was incubated overnight with 25µl of the PrG-Dynabeads (Sigma) and 1-5 µg of antibodies in 1ml reaction solution. pan H3 antibodies were used in quantity 1,5 µl per reaction (17-10254, Millipore), Ac-K9H3 5 µg (17-658, Millipore), diMe-K9H3 4 µg (ab-1220, abcam) and IgG rabbit as a negative control 2 µg (Millipore). Extracted DNA was rev-crosslinked, washed with the appropriate buffers from the Active Motif ChIP-IT Express kit and purified by phenol-chlorophorm extraction. After amplification, DNA samples were hybridized to the two-colored SurePrint G3 Human Promoter Microarray, 1x1M (G4873A, Agilent, Palo Alto, Calif., USA) covering gene promoter zones all over the genome. Labeling, hybridization and washing were carried out according to the Agilent mammalian ChIP-chip protocol (ver.9.0).

ChIP-on-chip Data Analysis

Scanned images were quantified with Agilent Feature Extraction software under standard conditions. The probe signals were filtered: replicated probes were merged by median and saturated probes (at least 1 channel) with high pixel heterogeneity have been removed. Filtered probes were processed as following: intra-array quantile normalization, log2 ratio transformation (ratio of modified histone probe signal to pan-H3 histone probe signal), GC%

normalization, Z-score transformation to homogenize the value distributions (median was subtracted from each log2(ratio) and result was divided by the standard deviation).

The p-value for each probe was computed by a modification of the Whitehead algorhythm as follows: an average was calculated for each probe with 2 surrounding it probes within 300bp, the distribution of these averages was obtained, each average value was reported to the distribution of averages. The area to the right of the value under the averages distribution curve was computed: this is the p-value which was attached to each probe. The threshold is set at 95% of the distribution (p-value = 0.05). The resulting output contains treated p-values ($-\log_{10}$(p-value)) with corresponding chromosome coordinates. We have imported these results into the Integrated Genome Browser [24] for visualization.

RT-qPCR

The expression level of 11q13 genes was determined by RT-qPCR using specific primers (**Table 1**). 100 ng of total RNA purified using guanidine thiocyanate and purification columns (NucleoSpin RNA II kit, Machery-Nagel) was converted into cDNA using Random Hexamer Primer (Fermentas) and RevertAid H Minus Reverse Transcriptase (Fermentas). cDNA was quantified using qPCR with FaStart Universal SYBR Green Master (Roche Diagnostics). Expression was calculated with ΔCt method (*GAPDH* gene expression used as the control). All values represent means±SEM of at least three biological replicates and follow a normal distribution. Statistical significance of the differences between gene expression values in MCL vs. control was estimated with unpaired Student's *t* test. For evaluation of statistically significant differences between untreated, 1h and 24h treated cells gene expression values, one-way ANOVA followed by Turkey post-test was applied.

RESULTS

Analysis of gene expression in the translocated 11q13 region in MCL cells reveals upregulated genes adjacent to the translocation point on chromosome 11.

We have first wanted to determine whether the t(11;14) perturbs expression of genes other than *CCND1* in the vicinity of the translocation point. In order to identify this, we have chosen 10 cancer-related genes located in the vicinity of the translocation point both on der 11 and der 14 chromosomes and studied their expression in five non-cancerous lymphoblastoid B cell lines (RPMI-8866, Priess, Remb1, IARC-211, IARC-171) and five MCL lines (Granta-519, Jeko-1, UPN-1, Mino and NCEB-1) using RT-qPCR analysis. These genes included *CD6* and *CD5*, T-lymphocyte surface antigens that play a role in T-cell activation and differentiation; *MTA2*, a component of the chromatin remodeling and histone deacetylase complex; *BAD*, a pro-apoptotic protein; *KAT5*, a MYST family histone acetylase; *CTSF*; a cysteine proteinase and a pro-apoptotic component of the lysosomal proteolytic system; *GSTP1*, a member of detoxication system of a cell; *CCND1*, a regulator of cell cycle G1/S transition; *ORAOV1*, a protein apparently involved in ribosome biogenesys; and *UVRAG*, a regulator of intracellular membrane trafficking and autophagy (**Table 2** and **Figure 1A**).

Most of the studied genes were overexpressed in MCL cells as compared to the controls; six genes (*CD6*, *CD5*, *CTSF*, *GSTP1*, *CCND1*, *ORAOV1*) showed a significant overexpression in MCL. Interestingly, five of them are either unexpressed or weakly expressed (*CD6*, *CD5*, *CTSF*, *ORAOV1* and *CCND1*) in normal cells (as compared to expression level of a housekeeping gene *GAPDH*). Three out of four genes that did not show a significant overexpression in MCL had a relatively high expression level in normal cells as compared to *GAPDH* (**Figure 1A**). Expression patterns of the individual cell lines are presented in the **Supplementary Figure 1**.

Globally, gene expression profiles were quite similar across MCL and across normal cell lines. In all cell lines *MTA2*, *KAT5*, *GSTP1*, and *UVRAG* genes showed a relatively high expression:

they were expressed stronger than the house-keeping gene *GAPDH*, and *GSTP1* was the most expressed (**Supplementary Figure 1**). In MCL *CCND1* showed the highest level of expression. In all tested control cell lines, *CD5* expression was not detectable; *CCND1* expression could not be detected neither in RPMI-8866 nor IARC-211; in three other control cells lines, *CCND1* was expressed at a very low level (**Supplementary Figure 1**).

Therefore, we have identified a set of chromosome 11 genes which were significantly overexpressed in MCL. These genes span over 15 Mb and can be found both on der11 (*CD6*, *CD5*, *GSTP1*, *CTSF*) and der14 (*CCND1*, *ORAOV1*). This global overexpression pattern can hardly be explained by the action of a single enhancer but rather could be a result of a large-scale post-translocation epigenetic regulation. We have next studied chromatin organization in several MCL and control cell lines.

Genes sensitive to upregulation after the translocation t(11;14) have different histone modification signature than the rest of genes in the 11q13 locus.

We have analyzed marks of active and inactive chromatin in genes' promoters using genome-wide ChIP-on-chip analysis in one control (RPMI-8866) and three MCL cell lines (Granta-519, Jeko-1, UPN-1). Chromatin was extracted from the cells and immunoprecipitated with antibodies against H3K9Ac (an active chromatin mark), H3K9me2 (a facultative heterochromatin mark) and panH3 as a reference. DNA extracted from the immunoprecipitated samples was used as a probe for hybridization with Agilent genomic microarrays covering gene promoters of human genome. The distributions of modified histone H3K9 in genes' promoters were compared to panH3. The statistical analysis was carried out as described in Material and Methods, and a number of statistically significant peaks of acetylation or di-methylation was calculated (p-value < 0.05). We have defined the level of histone H3K9 modifications separately for the whole genome, chromosome 11, the 11q13 locus harboring the translocation region, genes sensitive to upregulation (36; further referred as *upregulated*) and genes not-sensitive to upregulation genes (298; further referred as *not-upregulated*) in the 11q13 locus, detected previously.

At the genome level, the intensity of H3K9 acetylation and di-methylation were the same in the control and MCL cell lines, whereas in the 11q13 locus, H3K9 modification intensities differed between MCL and the control (**Figure 2**). Moreover, in both control and MCL cells, the *not-upregulated* genes had acetylation and di-methylation levels similar to that in the 11q13 locus, whereas *upregulated* genes had distinct profiles.

In the control cell line RPMI-8866, gene promoters in the 11q13 locus were acetylated and di-methylated twice less than in the rest of the genome, whereas *upregulated* genes were acetylated three times and di-methylated two times stronger than the 11q13 locus in general. All tested MCL cell lines were hyper-acetylated in the 11q13 locus as compared to the control (2-fold) and the rest of the genome. *Upregulated* genes had high level of acetylation in the control cell line, but in MCL cells acetylation level decreased: modestly (Jeko-1, UPN-1) or substantially (Granta-519).

A similar pattern was observed for H3K9me2 (with the exception of Granta-519): H3K9me2 level in the promoters in the 11q13 locus was twice higher in Jeko-1 and UPN-1 than in the control. *Upregulated* genes had high level of H3K9me2 in the control cell line, but in MCL cells acetylation level decreased: modestly (Jeko-1) or substantially (UPN-1).

Granta-519 had a different pattern of H3K9me2 distribution than other MCL cell lines. The general level of H3K9me2 in the entire Granta-519 genome and the 11 chromosome was lower than in other cell lines; in 11q13 locus, H3K9me2 level was low (twice lower than in other MCL cell lines); the upregulated genes had the lowest di-methylation level among all other tested cell lines. Increased acetylation and decreased methylation levels in the 11q13 locus in Granta-519 possibly indicate a particularly high level of chromatin activity in this region. Notably, seven genes out of ten 11q13 genes tested demonstrated the highest level of expression as compared to other MCL cell lines.

Thus, 11q13 locus had low H3K9Ac level in control cells; H3K9Ac increases twice after the translocation in MCL cell lines. In contrast, *upregulated* genes already had a high level of acetylation even in the control cells. After the translocation, this acetylation level decreased

slightly (Jeko-1, UPN-1) or substantially (Granta-519). A similar pattern of H3K9me2 distribution was observed. These data show that genes sensitive to upregulation after the translocation t(11;14) have a different histone modification signature than the rest of genes in the 11q13 locus. Moreover, after the translocation, this epigenetic signature changes in a cell line-specific way.

Next, we have studied the effect of an epigenetic drug abexinostat on chromatin structure and expression of the 11q13 genes in MCL.

Abexinostat induces heterochromatin disaggregation in normal and MCL cells

We have evaluated the global effect of the epigenetic drug abexinostat on heterochromatin in three control (RPMI-8866, Priess, IARC-211) and three MCL (Granta-519, NCEB-1, Jeko-1) cell lines. We have first evaluated the cytotoxic effect of abexinostat. Cell viability of abexinostat-treated cells was compared to cells incubated with 0.02% DMSO. Abexinostat induced 50% growth inhibition (GI50) at a dose of 0.02 µM in UPN-1 and Jeko-1 MCL cell lines at 24 hours (data not shown).

Cells were next treated with 100 nM abexinostat, fixed, stained with an antibody against a constitutive heterochromatin mark H3K9me3, and analyzed under the confocal microscope as described in Material and Methods. Large heterochromatin clusters were observed in non-treated cells. These clusters started to disintegrate already at 1h. At 24h, the global level of H3K9me3 dramatically decreased in all cell lines, and heterochromatin was organized in small clusters evenly distributed throughout the nucleus (**Figure 3**). No significant difference was observed between normal and MCL cells in this experiment. Thus, HDACi abexinostat induces global heterochromatin disaggregation both in normal and cancer cells.

Effect of abexinostat on 11q13 genes' expression in normal and MCL cell lines

We have then evaluated the effect of abexinostat on transcription of the 11q13 genes in the control and MCL cell lines. Five MCL and five control cell lines were treated with 100 nM abexinostat. The cells were collected at 1h post-treatment for the immediate effects and at 24h for the indirect effects mediated by the chromatin remodeling. RNA was isolated and gene expression was evaluated by RT-qPCR (**Figure 4**). While almost all tested genes had a tendency to increase their expression upon abexinostat treatment, some genes were strongly overexpressed (2-3 times) at 24h: *CD6*, *CTSF*, *GSTP1* and *CCND1* in the control cells; *CD6*, *CD5*, *CTSF* and *GSTP1* in MCL cell lines. Interestingly, most of the genes reacting to abexinostat were upregulated in MCL as compared to the control and had a relatively low level of expression (their expression was lower than that of *GAPDH*), with the exception of *GSTP1* (**Figure 1**). A very limited effect of abexinostat on transcription was observed at 1h post-treatment.

Thus, despite the global chromatin activation triggered by abexinostat (**Figure 3**), only a part of genes reacted to the treatment.

Promoters of the 11q13 genes are protected from the direct effect of abexinostat.

Next, we have analyzed *in fine* the effect of HDACi abexinostat on histone H3K9 modifications in genes' promoters using genome-wide ChIP-on-chip analysis. One control (RPMI-8866) and three MCL cell lines (Granta-519, Jeko-1, UPN-1) were treated with abexinostat for 1 and 24h, then chromatin was extracted from treated and non-treated cells and analyzed as described in the Materials and Methods.

Surprisingly, the HDAC inhibitor treatment did not induce hyperacetylation in genes' promoters in all tested cell lines on the levels of the entire genome, chromosome 11 and the 11q13 locus (with exception of UPN-1). Remarkable changes in histone modification levels were observed only in the promoters of genes sensitive to upregulation by the translocation (**Figure 5**). In the control RPMI-8866 cell line, acetylation in the promoters of the sensitive genes increased

modestly at 1h of abexinostat treatment, and then decreased at 24h. In Granta-519 cell line, acetylation increased more than two-fold at 1h of treatment, and then decreased to the initial level at 24h. Jeko-1 showed a two-fold decrease in H3K9Ac levels at 24h. UPN-1 cell line demonstrated a progressive decrease of acetylation both, in the entire 11q13 locus and in the set of *upregulated* genes.

Interestingly, H3K9me2 levels changed similarly to H3K9Ac upon abexinostat treatment in the *upregulated* genes in RPMI-8866, Granta-519 and Jeko-1 cell lines, and in the entire 11q13 locus in UPN-1. In the control cell line, H3K9me2 demonstrated a moderate progressive increase. In Granta-519, H3K9me2 level had the same changes as H3K9Ac: it increased more than three-fold at 1h of treatment, and then decreased two-fold at 24h. In Jeko-1 cells, H3K9me2 levels decreased progressively during the treatment. In UPN-1, H3K9me2 decreased modestly in the *upregulated* genes or dramatically in the 11q13 locus at 1h of treatment, and then at 24h, increased back to the initial level (11q13 locus). Thus in UPN-1, acetylation in the *upregulated* genes reacted more intensively to the treatment than in the *not-upregulated* genes, whereas methylation, changed stronger in the *not-upregulated* genes than in the *upregulated*.

DISCUSSUION

We and others have previously shown that chromosomal translocations are accompanied by the global relocalization of genes in the nucleus [8, 9]. This may lead to generalized upregulation of large gene clusters. We have selected ten cancer-related genes in the 11q13 locus situated in the vicinity of the translocation region and studied their expression in a panel of five non-cancerous lymphoblastoid cell lines and five MCL cell lines. Six of these genes were found to be upregulated in all MCL cell lines tested (**Figure 1**). Surprisingly, these genes were located on both sides of the translocation region, i.e. both on der11 and der14 chromosomes. Most of these genes, with the exception of *GSTP*, were either unexpressed or weakly expressed in normal B cells.

We have studied the effect of the HDAC inhibitor abexinostat on expression of these genes. Time points 1h and 24h post-application were chosen in order to distinguish between the immediate action of the drug and an indirect action which may be mediated by the induced changes in chromatin structure. A limited effect of abexinostat was observed at 1h, while different subsets of genes changed their expression at 24h in abexinostat-treated cells: *CD6*, *CTSF*, *GSTP1* and *CCND1* were overexpressed in the control cells while CD6, *CD5*, *CTSF* and *GSTP1* were overexpressed in MCL cell lines, though all genes revealed a tendency for increase of their expression. Some variations in this pattern were observed between different MCL or control cell lines (**Supplementary Figure 2**). Interestingly, most genes reacting on abexinostat treatment were upregulated in MCL as compared to the control, and they had a relatively low level of expression in the control cells. For example, abexinostat increased *CCND1* expression levels in MCL cells, but not in the control.

Next, we have studied the chromatin organization in abexinostat-treated and control cells. Large-scale movements of chromatin after the translocation may provoke global changes in histone modifications of chromatin in the 11q13 locus. Indeed, changes in the chromatin organization in MCL cells as compared to normal lymphocytes have been detected earlier [8, 25]. We have first studied the global organization of heterochromatin in the control and abexinostat-treated cells by immunofluorescence microscopy at 1 hour and 24 hours after treatment with abexinostat. The levels of the heterochromatin mark H3Kme3 dramatically decreased in both, control and MCL cell lines. These changes were associated with disappearance of peripheral heterochromatin clusters and redistribution of heterochromatin in cells with formation of an uniform punctuate pattern of heterochromatin **(Figure 3)**.

We have then used ChIP-on-chip to analyze changes induced by abexinostat in details. Surprisingly, while abexinostat had a global effect on chromatin structure in general, the genes themselves seemed to be shielded from its direct influence. Abexinostat triggered small changes in the H3K9Ac status of gene promoters. Acetylation did not simultaneously increase everywhere in the genome as it would be expected knowing the non-selective effect of HDACi. Only a small subset of genes from the entire genome reacted to abexinostat treatment, notably,

genes sensitive to upregulation after t(11;14) translocation in case of RPMI-8866, Granta-519, Jeko-1, and genes of entire 11q13 locus in UPN-1. The first effect of abexinostat (1h) on H3K9Ac levels was cell line-dependent, whereas the long-term effect (24h), decrease of H3K9 acetylation, was similar among all tested cell lines. Changes in the H3K9me2 patterns varied across different cell lines. This data indicates that, in general, gene promoters are protected from global changes triggered by the histone deacetylase inhibitor, and observed changes in histone modification levels hint on indirect mechanism of HDACi action.

A similar effect of chromatin-modifying agent on chromatin organization has been found in [26]. We showed that only genes sensitive to upregulation by t(11;14) translocation (or the entire 11q13 locus in UPN-1 cell line) showed intensive changes in their histone acetylation status (**Figure 5**). Methylation status of these genes as well reacted on the treatment. These data point to an indirect mechanism of abexinostat action.

CONCLUSIONS

Translocation (11;14) leads to upregulation of a cluster of genes located in the 11q13 locus on both sides of the translocation point. H3K9 acetylation status of this locus is elevated as compared to the average genome acetylation level. Regardless of a general heterochromatin disaggregation in response to abexinostat treatment, only a small subset of genes reacts to the treatment. Genes sensitive to upregulation after t(11;14) paradoxically decrease the level of acetylation in their promoters at 24h, though expression of some of these genes increases. Thus, genes mostly are sheltered from global changes triggered by abexinostat.

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CONFLICT OF INTEREST

The authors declare no conflict of interests

FIGURE LEGENDS

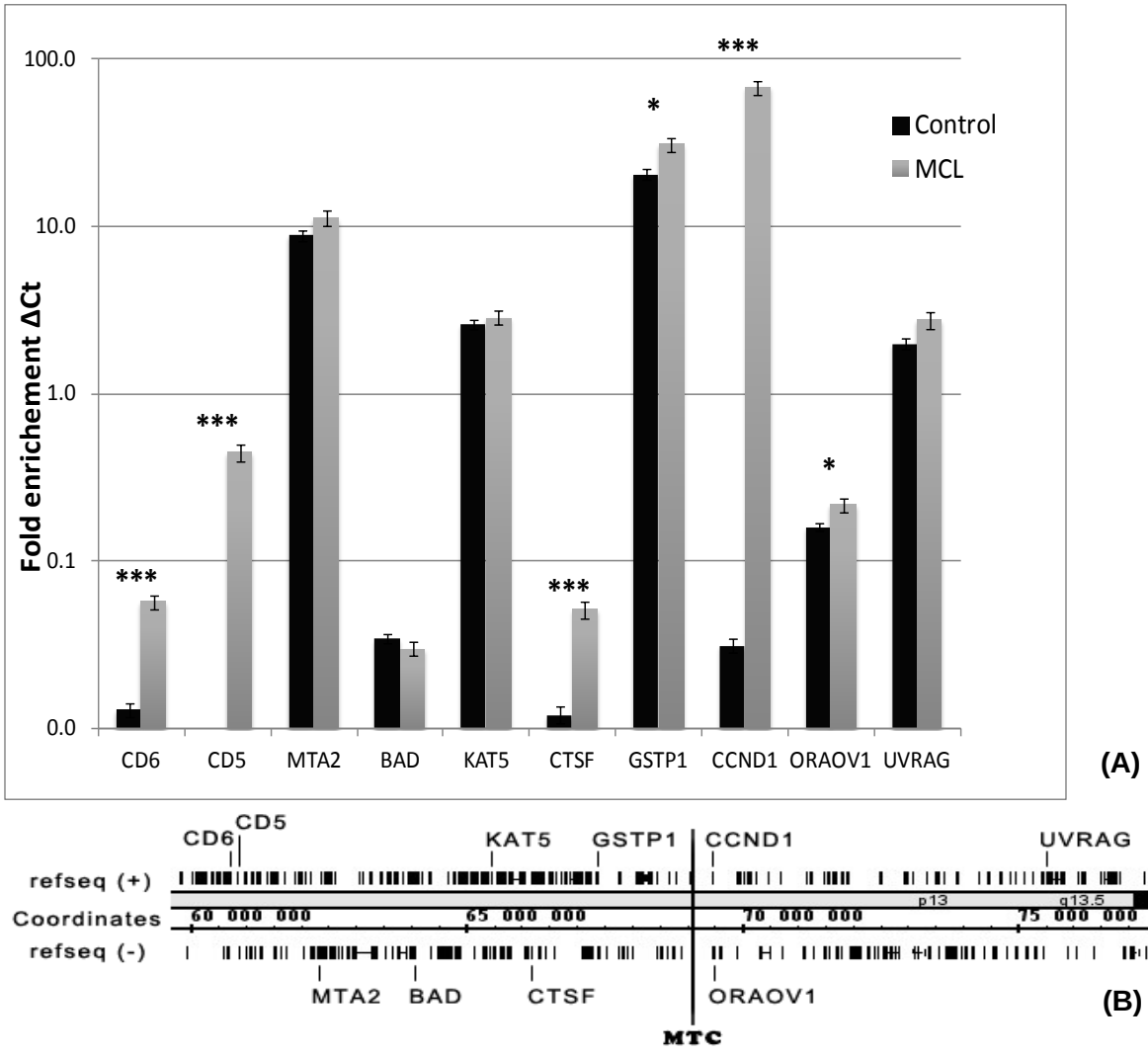


Figure 1. Gene expression levels of selected genes around the (11;14) translocation point. (**A**) The graphic represents average gene expression levels measured by RT-qPCR of 5 control cell lines (black) and 5 MCL cell lines (grey). Transcript abundance was normalized *GAPDH*, and presented on a base 10 logarithmic scale. The value 1 corresponds to *GAPDH* expression. At least 3 independent experiments were carried out for each of cell lines. The values are presented as mean±SEM. *p<0.05; ***p<0.001 (unpaired Student's *t* test relative to control) (**B**) Location of selected genes on the chromosome 11 relative to the translocation point (MTC – Major Translocation Cluster).

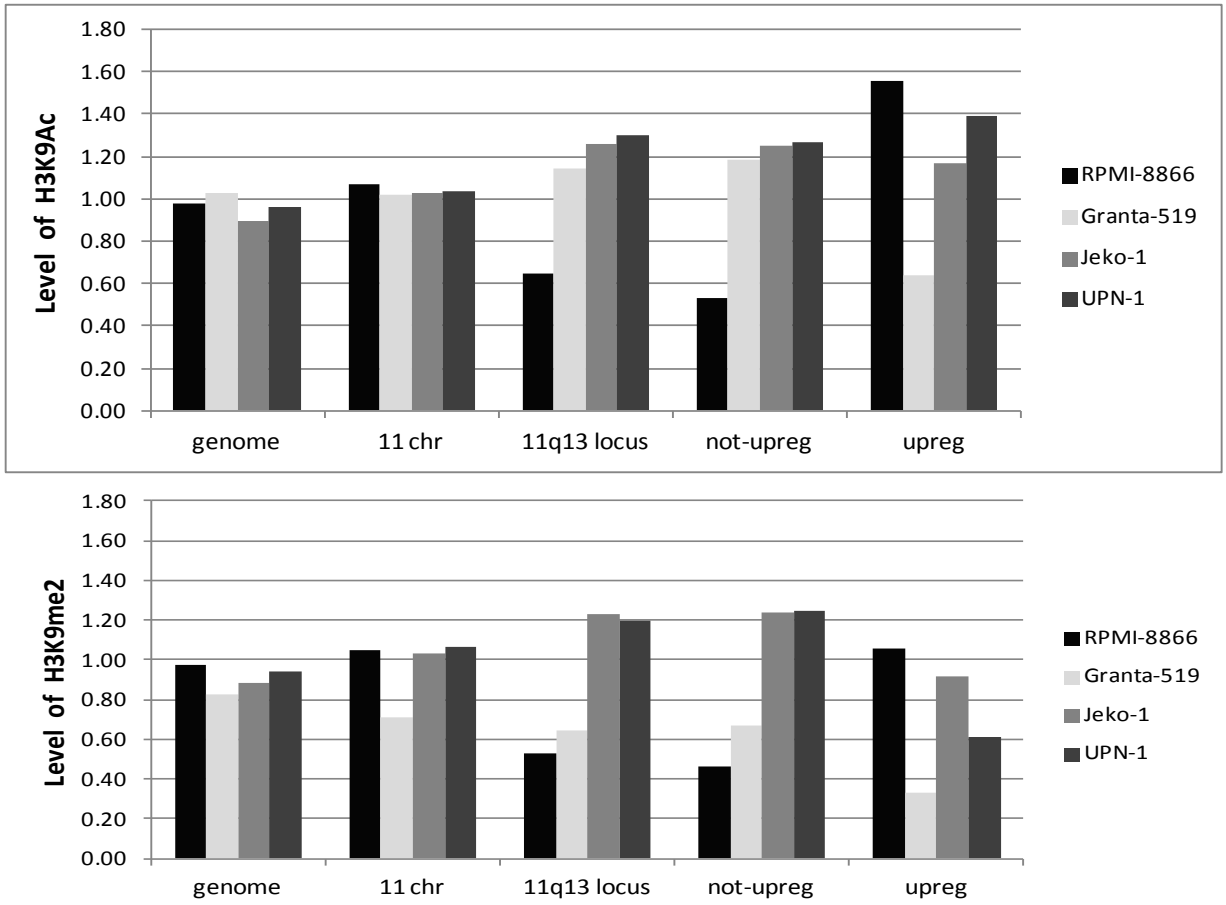


Figure 2. Level of H3K9 acetylation and H3K9 di-methylation in promoters of different subsets of genes. Chromatin from control cell line (RPMI-8866) and three MCL cell lines (Granta-519, Jeko-1, UPN-1) was immunoprecipitated with antibodies against H3K9Ac, H3K9me2 and panH3 as a reference. Enrichment in acetylation and methylation normalized to panH3 was estimated using Agilent Human Promoter Microarray. Statistically significant H3K9Ac and H3K9me2 peaks were calculated for the entire genome, the 11 chromosome, the 11q13 locus, for the genes which do not change their expression after the translocation (not-upreg) and for the genes sensitive to upregulation after the translocation t(11;14) (upreg). The data is presented as acetylation / methylation level (amount of statistically significant histone modification peaks divided by number of genes in the region analyzed).

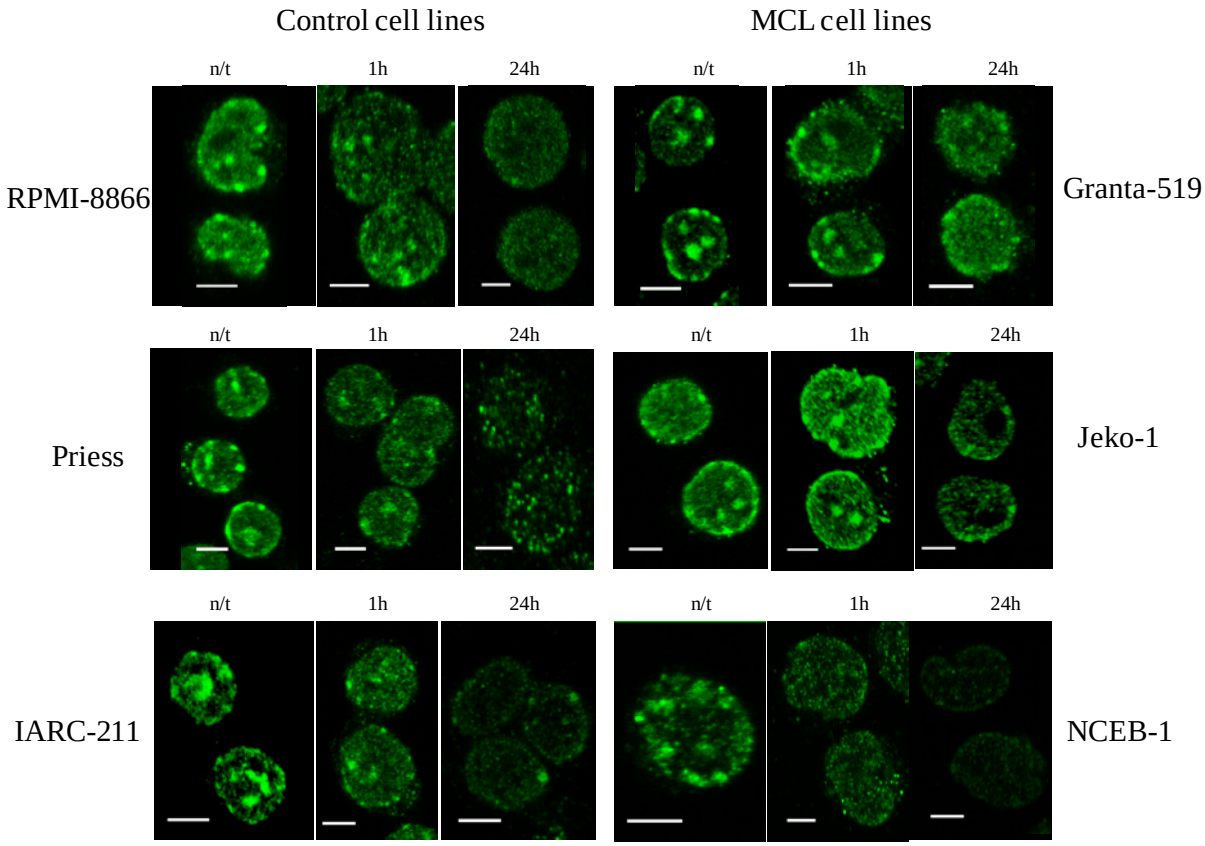


Figure 3. Changes in H3K9me3 levels in the control and MCL nuclei upon abexinostat treatment. Abexinostat treated for 1 and 24 hours and untreated cells (n/t) were fixed and immunostained for H3K9me3 (green). Scale bar = 5 μM

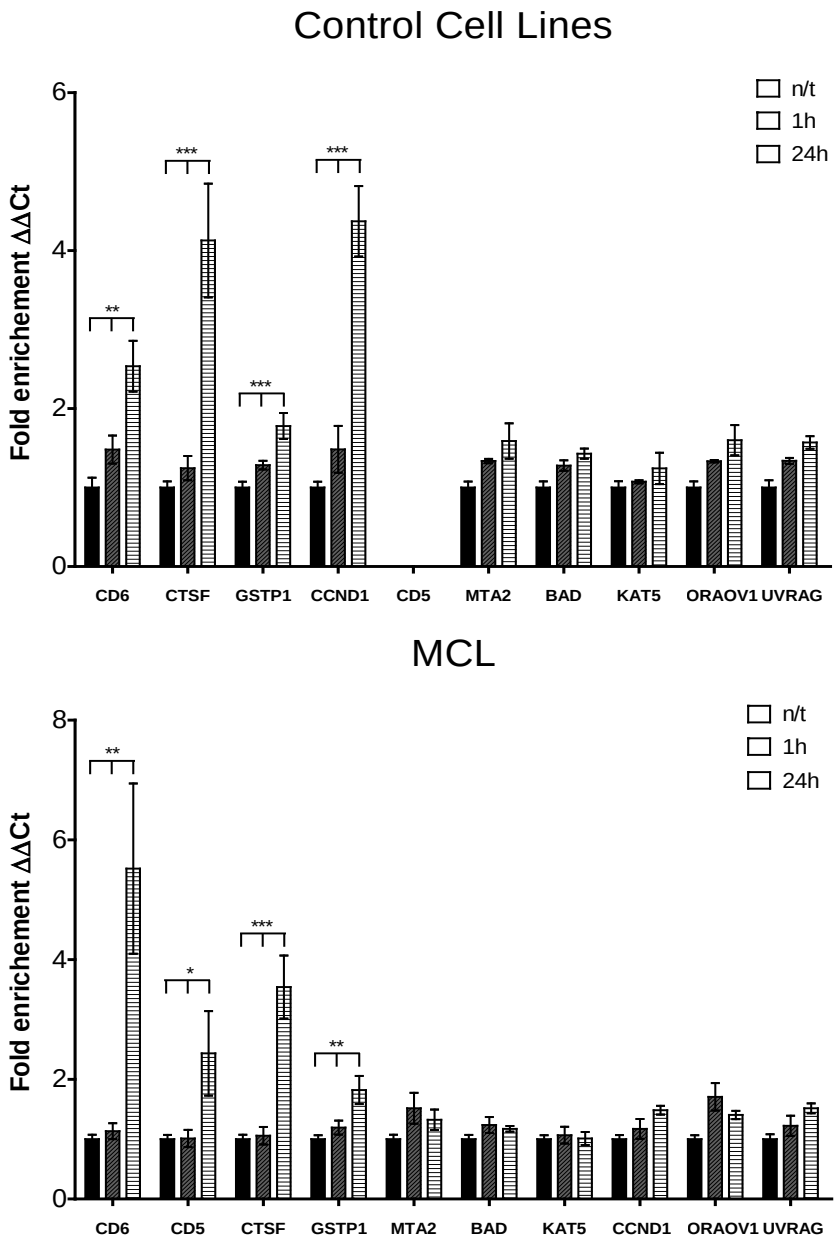


Figure 4. Effect of abexinostat on gene expression levels of 11q13 genes. Five MCL (Granta-519, Jeko-1, UPN-1, Mino and NCEB-1) and five control (RPMI-8866, Priess, Remb1, IARC-211, IARC-171) cell lines were treated with 100 nM abexinostat and the gene expression levels were assayed before the treatment (n/t, black), at 1h (grey) and 24h (white) after treatment. The expression level was measured by RT-qPCR vs. *GAPDH* expression. The data represent the average of 5 MCL and 5 control cell lines. At least 3 independent experiments for the each cell line were performed. The values are presented as mean±SEM. *p<0.05; **p<0.01; ***p<0.001 (1 way ANOVA with Turkey post-test).

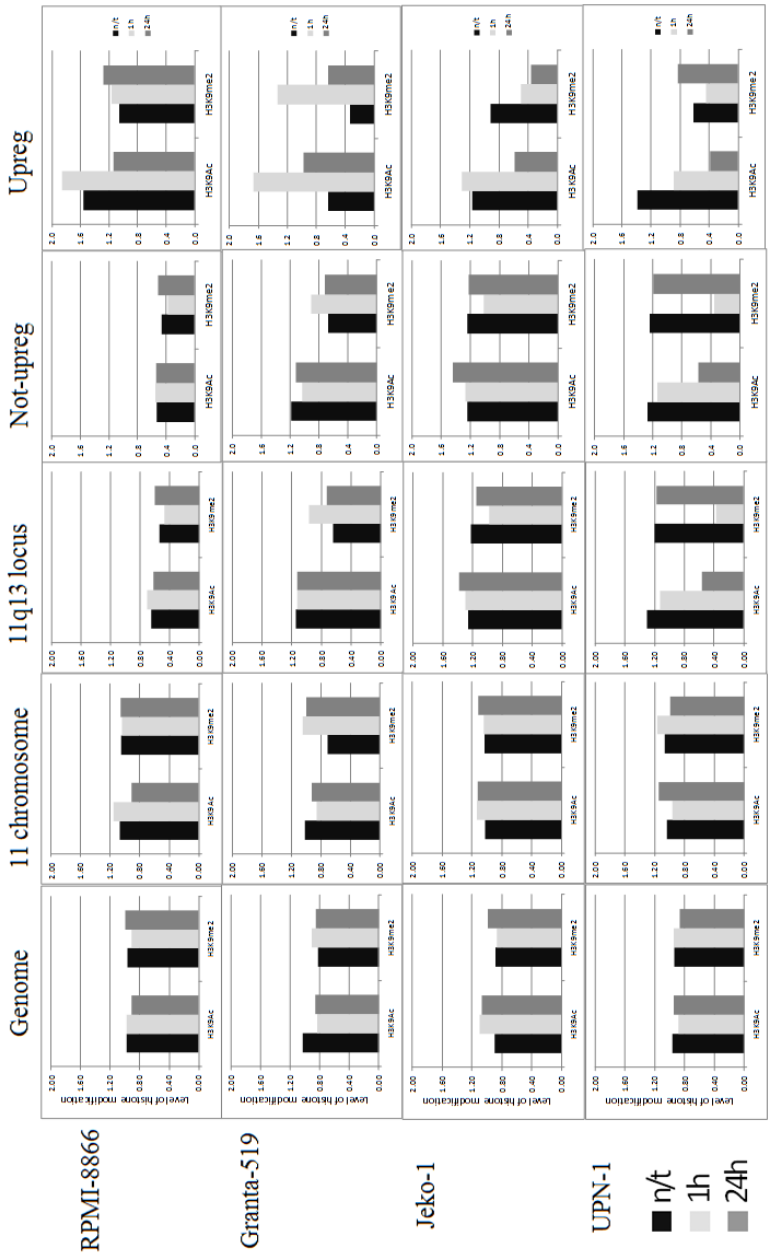
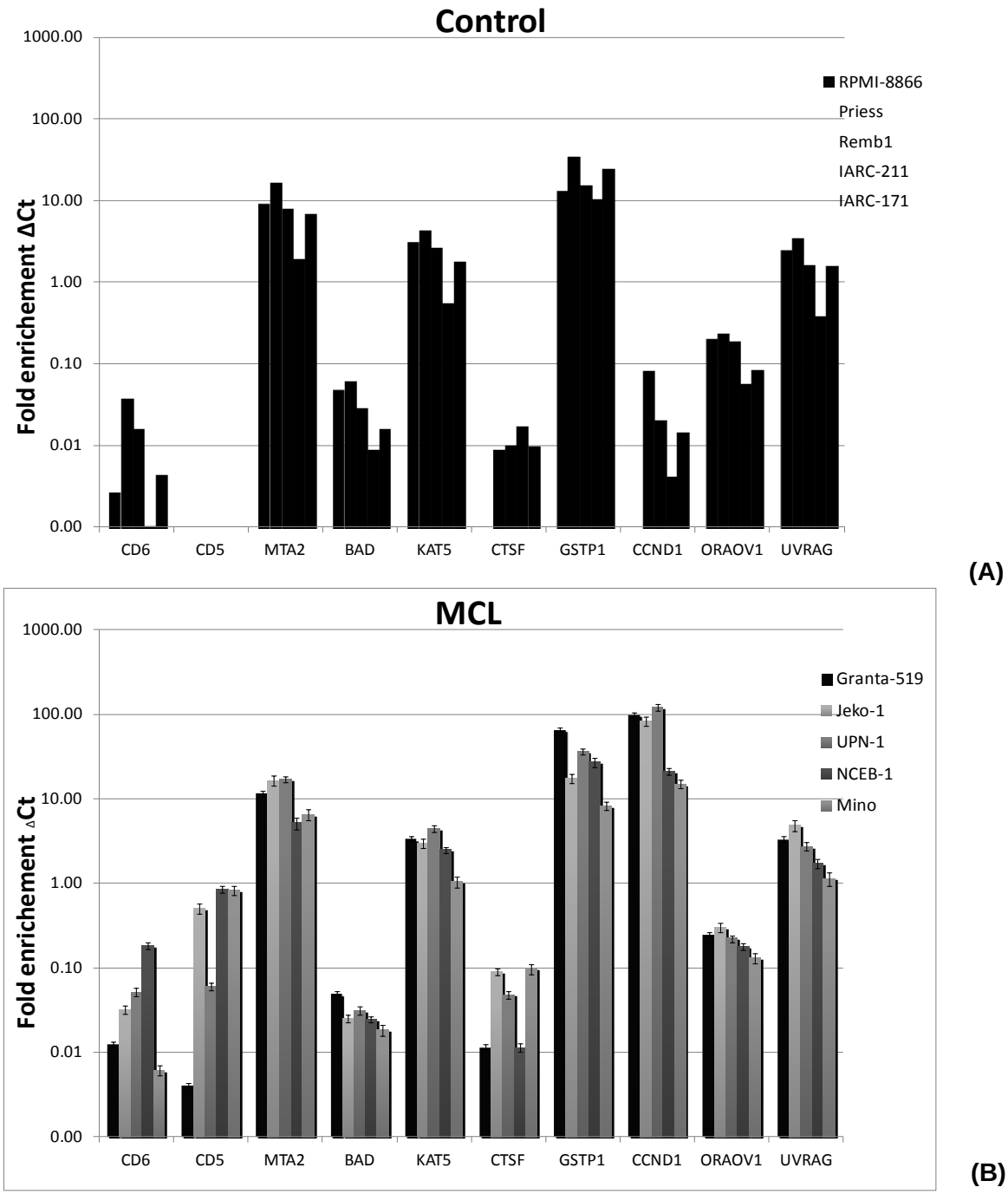
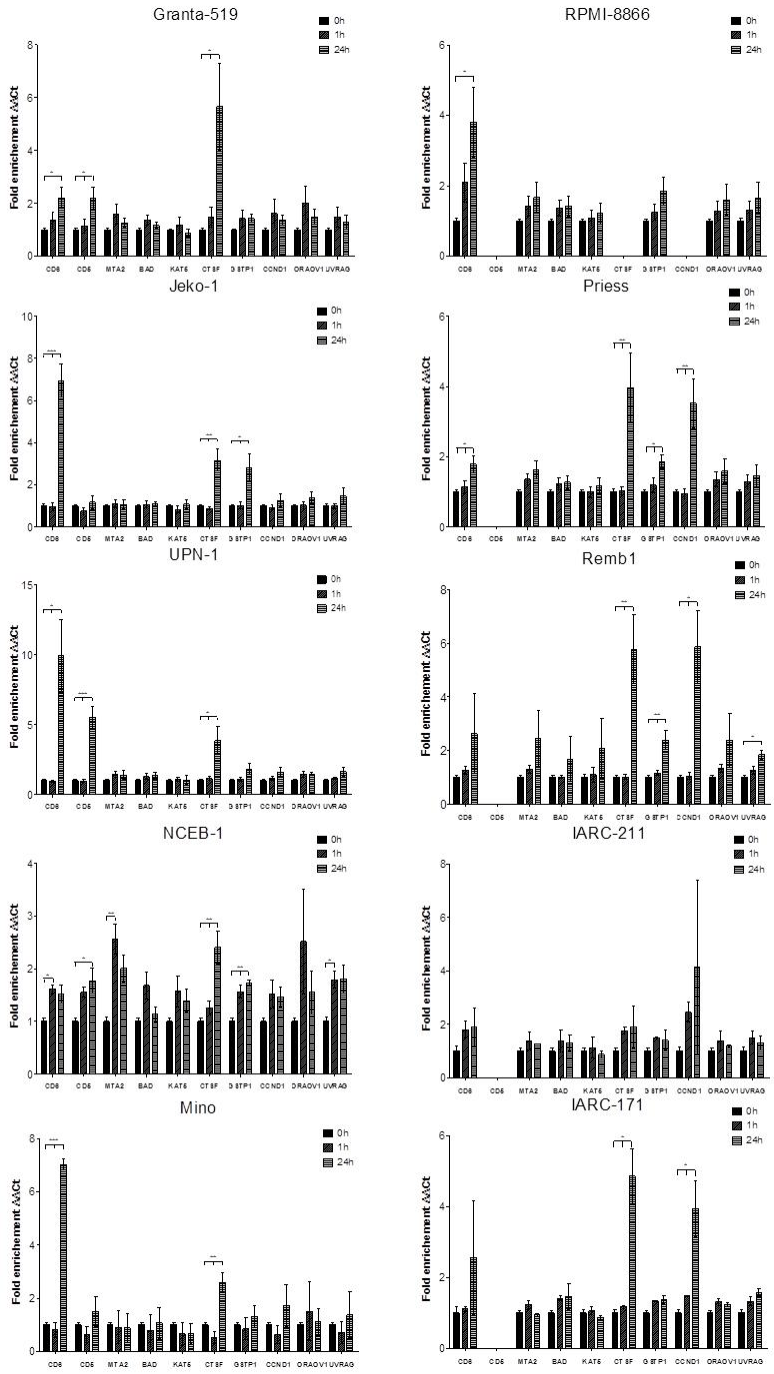


Figure 5. Changes in H3K9 acetylation and di-methylation levels in genes' promoters induced by abexinostat. MCL (Granta-519, Jeko-1, UPN-1) and control (RPMI-8866) cells were treated with 100 nM abexinostat and H3K9Ac, H3K9me2 enrichment normalized to panH3 was analyzed at defined time points using Agilent Human Promoter Microarray. The data is presented as acetylation / methylation level (amount of statistically significant histone modification peaks divided by number of genes in the region analyzed) in the entire genome, the 11 chromosome, the 11q13 locus, the genes which do not change their expression after the translocation (not-upreg) and the genes sensitive to upregulation after the translocation t(11;14) (upreg). *n/t* (black) – cells without treatment; *1h* (grey), *24h* (dark grey) – time points of abexinostat treatment.



Supplementary Figure 1. Individual gene expression patterns for 10 selected 11q13 genes around the (11;14) translocation point. The graphic represents gene expression levels measured by RT-qPCR of 5 control cell lines (**A**) and 5 MCL cell lines (**B**). Transcript abundance was normalized *GAPDH*, and presented on a base 10 logarithmic scale. The value 1 corresponds to *GAPDH* expression. At least 3 independent experiments were carried out for each of cell lines.



Supplementary Figure 2. Changes in transcript level induced by abexinostat in individual cell lines. The cells were treated with 100 nM abexinostat and the gene expression levels were assayed before the treatment (0h, black), at 1h (grey) and 24h (white) after treatment. The expression level was measured by RT-qPCR vs. *GAPDH* expression in 5 MCL and 5 control cell lines. The data represent the average of at least 3 independent experiments for each cell line. The values are presented as mean±SEM. *p<0.05; **p<0.01; ***p<0.001 (1 way ANOVA with Turkey post-test).

Table 1. RT-qPCR primers for cDNA

Gene		Sequence 5' - 3'
CD6	Fw	GCC CTG ACC ACC TTC TAC AGT
	Rv	GGG TTG GCA GTT GGG ATG T
CD5	Fw	CCA TCC GTC CTT GAG GTA GA
	Rv	CCT TGT ACC TGC TGG GGA T
MTA2	Fw	TAT GTG GGT GGC TGG TAA TG
	Rv	GCC TGG CTG ATA GTA ATG CC
BAD	Fw	TCA CCA GCA GGA GCA GCC AA
	Rv	GAG CGC GAG CGG CCC CGA AA
KAT5	Fw	CTT GGC CAA AAG ACA CAG GT
	Rv	CAT CCT CCA GGC AAT GAG AT
CTSF	Fw	GAC TGT GAC AAG ATG GAC AA
	Rv	CCA CGG AGT CAT TGA TGT AGA
GSTP1	Fw	AAT GAA GGT CTT GCC TCC CT
	Rv	GAC CTC CGC TGC AAA TAC AT
CCND1	Fw	AGT TGT TGG GGC TCC TCA G
	Rv	AGA CCT TCG TTG CCC TCT GT
UVRAG	Fw	TGG AGT CCC TAG TCC ATG TTG
	Rv	AGG AGG GGA GAA GTT GCA GT
ORAOV1	Fw	GTC AGG ACA TAT TCG ATG CCA T
	Rv	GCT GCC TTC CCT CCA TCA CA
GAPDH	Fw	CTG CAC CAC CAA CTG CTT AG
	Rv	AGG TCC ACC ACT GAC ACG TT

Table 2. Properties of the selected genes in 11q13 locus and their expression in control and MCL cell lines

Gene	Expression rate MCL vs. control[#]	Function	Expression in cancer	References
CD6	4.4	Lymphocyte glycoprotein receptor on the majority of T cells and a subset of B cells. Mediates cellular adhesion migration across the endothelial and epithelial cells. Participates in the antigen presentation by B cells and the subsequent proliferation of T cells	Prostate cancer. T cell large granular lymphocyte leukemia. Centrocytic lymphoma.	[27] [28] [29] [30]
CD5	∞ [##]	Lymphocyte glycoprotein receptor on T cells and in small proportion on B cells that signals cell growth.	Blastic mantle cell lymphoma cells. T-cell leukemia/lymphoma. Chronic lymphocytic B-cell leukemia. Thymic sarcoma.	[31] [32] [33]
MTA2	1.3	Component of NuRD, a chromatin remodeling and histone deacetylase complex. Strongly expressed in many tissues.	Ovarian epithelial cancer. Breast tumor. B cell acute lymphoblastic leukemia. Adenocarcinoma. Gastric cancer.	[34] [35] [36] [37] [38]
BAD	0.9	Pro-apoptotic protein positively regulating cell apoptosis by forming heterodimers with BCL-xL and BCL-2, and reversing their death repressor activity.	Down-regulated in breast and ovarian cancers.	[39] [40] [41]
KAT5	1.1	Histone acetylase from the MYST family. Plays a role in DNA repair, apoptosis and in signal transduction.	Overexpression in melanoma associated with increased chemosensitivity. Down-regulation is associated with malignancy of gastric, colon, lung, pancreatic, breast, metastatic melanoma cancers.	[42] [43] [44] [45] [46]

CTSF	4.3	Cathepsin F is a component of the lysosomal proteolytic system. Ubiquitously expressed.	Cervical cancer. Breast cancer.	[47] [48]
GSTP1	1.5	Plays an important role in detoxification and catalyzes detoxification of xenobiotics including carcinogens via conjugation to glutathione.	Lack of expression in human prostate cancer cells. Upregulated in neoplastic cells, non-small cell lung cancer. Associated with drug resistance.	[49] [50] [51] [52] [53]
CCND1	2154.6	Regulator of CDK4 or CDK6 kinases, required for cell cycle G1/S transition.	Mantle cell lymphomas and t(11q13)-associated leukemias. Carcinoma. Breast cancer. Non-small-cell lung cancer. Colorectal cancer. Melanoma. Multiple myeloma.	[54] [55] [56] [57]
ORAOV1	1.3	Plays essential roles in the function and biogenesis of the ribosome.	Esophageal carcinoma. Gastric adenocarcinoma. Squamous cell carcinomas. Cervical cancer. Oral cancer.	[58] [59] [60] [61] [62]
UVRAG	1.4	Critical regulator of intracellular membrane trafficking, including autophagy and chromosomal stability. Tumor suppressor.	Deleted, mutated or dowmregulated in colon, breast and gastric cancers.	[63] [64] [65] [66]

[#] - Expression was measured using RT-qPCR and presented as average of fold-enrichement ΔCt [target - ref gene (*GAPDH*)] in 5 MCL relative to 5 control cell lines. At least 3 independent experiments were performed for each cell line.
[##] - *CD5* was not expressed in normal lymphoblastoid cell lines.

Table 3. Abexinostat-induced changes in H3K9 acetylation and di-methylation levels in gene promoters

Cell line	Condition	Genome		11 chromosome		11q13 locus	
		H3K9Ac	H3K9me2	H3K9Ac	H3K9me2	H3K9Ac	H3K9me2
RPMI-8866	**n/t**	0.98	0.97	1.07	1.05	0.65	0.53
	1h	0.97	0.91	1.15	1.04	0.70	0.47
	24h	0.91	0.99	0.91	1.06	0.62	0.60
Granta-519	**n/t**	1.03	0.82	1.02	0.71	1.14	0.64
	1h	0.83	0.91	0.86	1.05	1.12	0.97
	24h	0.86	0.85	0.93	0.99	1.12	0.73
Jeko-1	**n/t**	0.90	0.88	1.03	1.04	1.26	1.23
	1h	1.10	0.87	1.14	1.04	1.30	0.98
	24h	1.07	0.98	1.12	1.11	1.38	1.16
UPN-1	**n/t**	0.96	0.94	1.04	1.06	1.30	1.20
	1h	0.88	0.94	0.96	1.16	1.13	0.37
	24h	0.94	0.86	1.14	0.99	0.57	1.18

MCL and control cells were treated with 100 nM abexinostat and H3K9Ac, H3K9me2 enrichment normalized to panH3 was analyzed at defined time points using Agilent Human Promoter Microarray. The data is presented as acetylation / methylation level (amount of statistically significant histone modification peaks divided by number of genes in the region analyzed) in the entire genome, the 11 chromosome and the 11q13 locus. n/t – cells without treatment; 1h, 24h – time points of abexinostat treatment.

REFERENCES

1. Schmidt, C. and M. Dreyling, *Therapy of mantle cell lymphoma: current standards and future strategies.* Hematol Oncol Clin North Am, 2008. 22(5): p. 953-63, ix.
2. Jaffe, E., et al., eds. *Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues*. World Health Organization Classification of Tumours. 2001, IARC Press: Lyon, France.
3. Wlodarska, I., et al., *Translocations targeting CCND2, CCND3, and MYCN do occur in t(11;14)-negative mantle cell lymphomas.* Blood, 2008. 111(12): p. 5683-90.
4. Greiner, T.C., et al., *Mutation and genomic deletion status of ataxia telangiectasia mutated (ATM) and p53 confer specific gene expression profiles in mantle cell lymphoma.* Proc Natl Acad Sci U S A, 2006. 103(7): p. 2352-7.
5. Smith, J., et al., *The ATM-Chk2 and ATR-Chk1 pathways in DNA damage signaling and cancer.* Adv Cancer Res, 2010. 108: p. 73-112.
6. Fiancette, R., et al., *A myeloma translocation-like model associating CCND1 with the immunoglobulin heavy-chain locus 3' enhancers does not promote by itself B-cell malignancies.* Leuk Res, 2010. 34(8): p. 1043-51.
7. Bennaceur-Griscelli, A., et al., *High level of glutathione-S-transferase pi expression in mantle cell lymphomas.* Clin Cancer Res, 2004. 10(9): p. 3029-34.
8. Allinne, J., et al., *Perinucleolar relocalization and nucleolin as crucial events in the transcriptional activation of key genes in mantle cell lymphoma.* Blood, 2014. 123(13): p. 2044-53.
9. Harewood, L., et al., *The effect of translocation-induced nuclear reorganization on gene expression.* Genome Res, 2010. 20(5): p. 554-64.
10. Rafique, S., et al., *Estrogen-induced chromatin decondensation and nuclear re-organization linked to regional epigenetic regulation in breast cancer.* Genome Biol, 2015. 16: p. 145.
11. Razin, S.V., et al., *Chromatin domains and territories: flexibly rigid.* Crit Rev Eukaryot Gene Expr, 2004. 14(1-2): p. 79-88.
12. Kosak, S.T., et al., *Subnuclear compartmentalization of immunoglobulin loci during lymphocyte development.* Science, 2002. 296(5565): p. 158-62.
13. Chambeyron, S. and W.A. Bickmore, *Chromatin decondensation and nuclear reorganization of the HoxB locus upon induction of transcription.* Genes Dev, 2004. 18(10): p. 1119-30.
14. Iarovaia, O.V., et al., *Dynamics of double strand breaks and chromosomal translocations.* Mol Cancer, 2014. 13: p. 249.
15. Camara-Clayette, V., O. Hermine, and V. Ribrag, *Emerging agents for the treatment of mantle cell lymphoma.* Expert Rev Anticancer Ther, 2012. 12(9): p. 1205-15.
16. Bolden, J.E., M.J. Peart, and R.W. Johnstone, *Anticancer activities of histone deacetylase inhibitors.* Nat Rev Drug Discov, 2006. 5(9): p. 769-84.
17. Xu, W.S., R.B. Parmigiani, and P.A. Marks, *Histone deacetylase inhibitors: molecular mechanisms of action.* Oncogene, 2007. 26(37): p. 5541-52.
18. Kawamata, N., J. Chen, and H.P. Koeffler, *Suberoylanilide hydroxamic acid (SAHA; vorinostat) suppresses translation of cyclin D1 in mantle cell lymphoma cells.* Blood, 2007. 110(7): p. 2667-73.
19. Dasmahapatra, G., et al., *Carfilzomib interacts synergistically with histone deacetylase inhibitors in mantle cell lymphoma cells in vitro and in vivo.* Mol Cancer Ther, 2011. 10(9): p. 1686-97.

20. West, A.C. and R.W. Johnstone, *New and emerging HDAC inhibitors for cancer treatment.* J Clin Invest, 2014. 124(1): p. 30-9.
21. Morschhauser, F., et al., *Phase 1 study of the oral histone deacetylase inhibitor abexinostat in patients with Hodgkin lymphoma, non-Hodgkin lymphoma, or chronic lymphocytic leukaemia.* Invest New Drugs, 2015.
22. Bhalla, S., et al., *PCI-24781 induces caspase and reactive oxygen species-dependent apoptosis through NF-kappaB mechanisms and is synergistic with bortezomib in lymphoma cells.* Clin Cancer Res, 2009. 15(10): p. 3354-65.
23. Solovei, I., et al., *FISH: A Practical Approach*, in *FISH: A Practical Approach*, J. Squire, B. Beatty, and S. Mai, Editors. 2002, Oxford Univ. Press: Oxford.
24. Nicol, J.W., et al., *The Integrated Genome Browser: free software for distribution and exploration of genome-scale datasets.* Bioinformatics, 2009. 25(20): p. 2730-1.
25. Liu, H., J. Wang, and E.M. Epner, *Cyclin D1 activation in B-cell malignancy: association with changes in histone acetylation, DNA methylation, and RNA polymerase II binding to both promoter and distal sequences.* Blood, 2004. 104(8): p. 2505-13.
26. Halsall, J., et al., *Genes are often sheltered from the global histone hyperacetylation induced by HDAC inhibitors.* PLoS One, 2012. 7(3): p. e33453.
27. Escoda-Ferran, C., et al., *Modulation of CD6 function through interaction with Galectin-1 and -3.* FEBS Lett, 2014. 588(17): p. 2805-13.
28. Alonso-Ramirez, R., et al., *Rationale for Targeting CD6 as a Treatment for Autoimmune Diseases.* Arthritis, 2010. 2010: p. 130646.
29. Daibata, M., et al., *Differential gene-expression profiling in the leukemia cell lines derived from indolent and aggressive phases of CD56+ T-cell large granular lymphocyte leukemia.* Int J Cancer, 2004. 108(6): p. 845-51.
30. Zukerberg, L.R., et al., *Diffuse low-grade B-cell lymphomas. Four clinically distinct subtypes defined by a combination of morphologic and immunophenotypic features.* Am J Clin Pathol, 1993. 100(4): p. 373-85.
31. Hishima, T., et al., *CD5 expression in thymic carcinoma.* Am J Pathol, 1994. 145(2): p. 268-75.
32. Kaplan, D., et al., *CD5 expression by B lymphocytes and its regulation upon Epstein-Barr virus transformation.* Proc Natl Acad Sci U S A, 2001. 98(24): p. 13850-3.
33. Liu, Z., et al., *CD5- mantle cell lymphoma.* Am J Clin Pathol, 2002. 118(2): p. 216-24.
34. Ji, Y., et al., *Expression of MTA2 gene in ovarian epithelial cancer and its clinical implication.* J Huazhong Univ Sci Technolog Med Sci, 2006. 26(3): p. 359-62.
35. Cui, Y., et al., *Metastasis-associated protein 2 is a repressor of estrogen receptor alpha whose overexpression leads to estrogen-independent growth of human breast cancer cells.* Mol Endocrinol, 2006. 20(9): p. 2020-35.
36. Chen, D.W., et al., *MTA2 expression is a novel prognostic marker for pancreatic ductal adenocarcinoma.* Tumour Biol, 2013. 34(3): p. 1553-7.
37. Covington, K.R. and S.A. Fuqua, *Role of MTA2 in human cancer.* Cancer Metastasis Rev, 2014. 33(4): p. 921-8.
38. Zhou, C., et al., *MTA2 promotes gastric cancer cells invasion and is transcriptionally regulated by Sp1.* Mol Cancer, 2013. 12(1): p. 102.
39. Cekanova, M., et al., *BCL-2 family protein, BAD is down-regulated in breast cancer and inhibits cell invasion.* Exp Cell Res, 2015. 331(1): p. 1-10.

40. Borhani, N.M., Mehdi; Gargari, S. Soraya; Novin, G. Marefat; Mansouri, Ardalan; Omrani, D. Mir *Decreased Expression of Proapoptotic Genes Caspase-8- and BCL2-Associated Agonist of Cell Death (BAD) in Ovarian Cancer.* Clinical Ovarian and Other Gynecologic Cancer, 2014. 7(1-2): p. 18-23.
41. Sastry, K.S., et al., *Targeting proapoptotic protein BAD inhibits survival and self-renewal of cancer stem cells.* Cell Death Differ, 2014. 21(12): p. 1936-49.
42. Chen, G., et al., *Role of Tip60 in human melanoma cell migration, metastasis, and patient survival.* J Invest Dermatol, 2012. 132(11): p. 2632-41.
43. Sakuraba, K., et al., *TIP60 as a potential marker for the malignancy of gastric cancer.* Anticancer Res, 2011. 31(1): p. 77-9.
44. Patani, N., et al., *Histone-modifier gene expression profiles are associated with pathological and clinical outcomes in human breast cancer.* Anticancer Res, 2011. 31(12): p. 4115-25.
45. Chevillard-Briet, M., et al., *Interplay between chromatin-modifying enzymes controls colon cancer progression through Wnt signaling.* Hum Mol Genet, 2014. 23(8): p. 2120-31.
46. Van Den Broeck, A., et al., *Activation of a Tip60/E2F1/ERCC1 network in human lung adenocarcinoma cells exposed to cisplatin.* Carcinogenesis, 2012. 33(2): p. 320-5.
47. Vazquez-Ortiz, G., et al., *Overexpression of cathepsin F, matrix metalloproteinases 11 and 12 in cervical cancer.* BMC Cancer, 2005. 5: p. 68.
48. Allinen, M., et al., *Molecular characterization of the tumor microenvironment in breast cancer.* Cancer Cell, 2004. 6(1): p. 17-32.
49. Lin, X., et al., *GSTP1 CpG island hypermethylation is responsible for the absence of GSTP1 expression in human prostate cancer cells.* Am J Pathol, 2001. 159(5): p. 1815-26.
50. Cumming, R.C., et al., *Fanconi anemia group C protein prevents apoptosis in hematopoietic cells through redox regulation of GSTP1.* Nat Med, 2001. 7(7): p. 814-20.
51. Rybarova, S., et al., *MRP1 and GSTp1 expression in non-small cell lung cancer does not correlate with clinicopathological parameters: A Slovakian population study.* Acta Histochem, 2014. 116(8): p. 1390-8.
52. Townsend, D.M. and K.D. Tew, *The role of glutathione-S-transferase in anti-cancer drug resistance.* Oncogene, 2003. 22(47): p. 7369-75.
53. Arai, T., et al., *Association of GSTP1 expression with resistance to docetaxel and paclitaxel in human breast cancers.* Eur J Surg Oncol, 2008. 34(7): p. 734-8.
54. Rimokh, R., et al., *Rearrangement of CCND1 (BCL1/PRAD1) 3' untranslated region in mantle-cell lymphomas and t(11q13)-associated leukemias.* Blood, 1994. 83(12): p. 3689-96.
55. Freier, K., et al., *Tissue microarray analysis reveals site-specific prevalence of oncogene amplifications in head and neck squamous cell carcinoma.* Cancer Res, 2003. 63(6): p. 1179-82.
56. Hosokawa, Y. and A. Arnold, *Mechanism of cyclin D1 (CCND1, PRAD1) overexpression in human cancer cells: analysis of allele-specific expression.* Genes Chromosomes Cancer, 1998. 22(1): p. 66-71.
57. Musgrove, E.A., et al., *Cyclin D as a therapeutic target in cancer.* Nat Rev Cancer, 2011. 11(8): p. 558-72.
58. Zhai, C., et al., *The function of ORAOV1/LTO1, a gene that is overexpressed frequently in cancer: essential roles in the function and biogenesis of the ribosome.* Oncogene, 2014. 33(4): p. 484-94.

59. **Li, M., et al.,** ***ORAOV1 overexpression in esophageal squamous cell carcinoma and esophageal dysplasia: a possible biomarker of progression and poor prognosis in esophageal carcinoma.*** **Hum Pathol, 2015. 46(5): p. 707-15.**
60. **Kang, J.U. and S.H. Koo,** ***ORAOV1 is a probable target within the 11q13.3 amplicon in lymph node metastases from gastric adenocarcinoma.*** **Int J Mol Med, 2012. 29(1): p. 81-7.**
61. **Jiang, L., et al.,** ***Oral cancer overexpressed 1 (ORAOV1) regulates cell cycle and apoptosis in cervical cancer HeLa cells.*** **Mol Cancer, 2010. 9: p. 20.**
62. **Huang, X., et al.,** ***High-resolution mapping of the 11q13 amplicon and identification of a gene, TAOS1, that is amplified and overexpressed in oral cancer cells.*** **Proc Natl Acad Sci U S A, 2002. 99(17): p. 11369-74.**
63. **He, S., et al.,** ***Truncating mutation in the autophagy gene UVRAG confers oncogenic properties and chemosensitivity in colorectal cancers.*** **Nat Commun, 2015. 6: p. 7839.**
64. **Ionov, Y., et al.,** ***Manipulation of nonsense mediated decay identifies gene mutations in colon cancer Cells with microsatellite instability.*** **Oncogene, 2004. 23(3): p. 639-45.**
65. **Kim, M.S., et al.,** ***Frameshift mutation of UVRAG, an autophagy-related gene, in gastric carcinomas with microsatellite instability.*** **Hum Pathol, 2008. 39(7): p. 1059-63.**
66. **Knaevelsrud, H., et al.,** ***UVRAG mutations associated with microsatellite unstable colon cancer do not affect autophagy.*** **Autophagy, 2010. 6(7): p. 863-70.**

4. Effect of abexinostat on gene expression in t(14;18) follicular lymphoma cells

Since abexinostat did not affect *CCND1* expression in t(11;14) mantle cell lymphoma, but led instead to overexpression of some weakly expressed genes (**Figure 1** and **Figure 4 in the manuscript**), we wanted to verify whether this effect could be observed in other types of lymphomas. For this, we assessed abexinostat effect on gene expression in follicular lymphoma cells. Follicular lymphoma is one of the most common types of non-Hodgkin's lymphomas harboring t(18;14) or t(3;14) translocations, which lead to *BCL-2* or *BCL-6* overexpression, respectively. A recent study demonstrated downregulation of BCL-2 protein in t(14;18) lymphoma by HDACi (Duan et al., 2005).

First, we have tested a follicular lymphoma cell line RL and one sample from a patient with follicular lymphoma for the expression of 3 genes in 11q13 locus (*CTSF*, *GSTP1*, *CCND1*) and of *BCL-2* gene on the 18 chromosome. Three independent experiments were performed for the RL cell line, and average of gene expression enrichment was calculated (**Figure 17**). As expected, *BCL-2* was overexpressed (290 times higher than *GAPDH* expression in the patient sample and 30 times higher in the RL cell line). *CTSF* and *CCND1* expressions were very low in the patient's sample and were not detectable in RL cell line. *GSTP1* was highly expressed, though in normal B-lymphocytes (NBL and LCLs) it showed overexpression too.

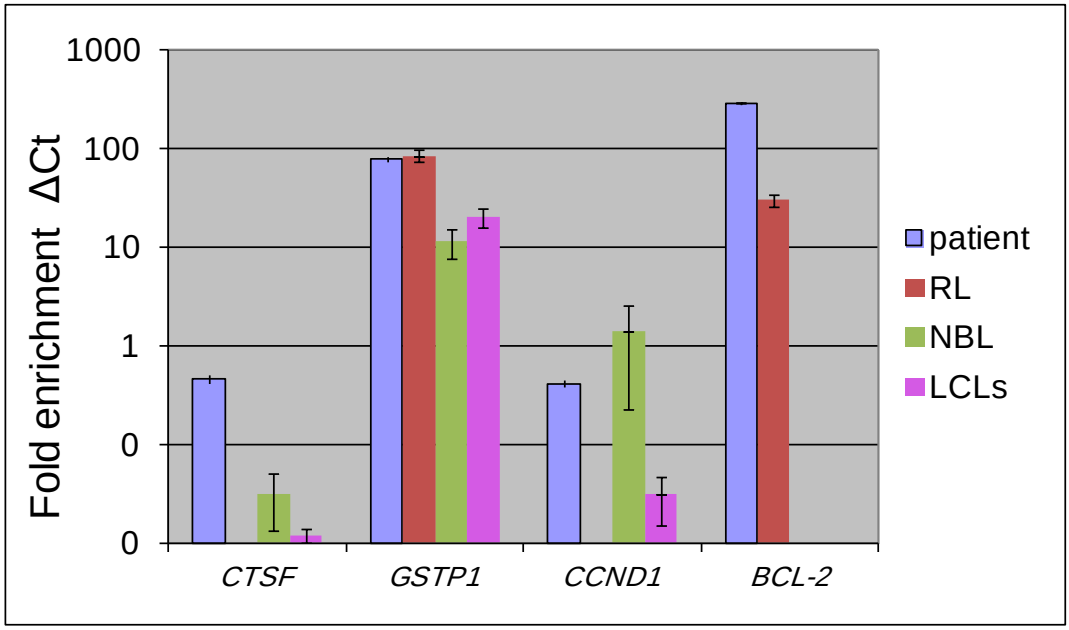


Figure 17. Gene expression in follicular lymphoma and normal cells.

Follicular lymphoma cell line RL, a sample from a patient with follicular lymphoma, normal B-lymphocytes from donors (NBL) and normal lymphoblastoid cell lines (LCLs) were tested for the gene expression by RT-qPCR. The data represents fold enrichment average for three independent experiments on RL cell line, for three NBL samples and for 6 normal cell lines (LCLs: RPMI-8866, Priess, Remb1, IARC-211, IARC-171). *BCL-2* expression in NBL and LCLs was not analyzed. Transcript abundance was normalized *GAPDH* (corresponds to the value 1), and presented on a base 10 logarithmic scale. The data is presented as mean±SEM.

Next, we have assessed effect of abexinostat on gene expression in follicular lymphoma. Cells were treated with 100 nM abexinostat for 1 and 24 hours, and then expression was analyzed in reference to *GAPDH* gene. In the patient's cells, expression was measured after 1h and 24h in both, treated and not treated cells, then values in treated cells were related to that from untreated cells from the same time point.

No influence of abexinostat was detected on *BCL-2* or *GSTP1* expression in both, patient's cells and RL cell line (**Figure 18**). Two genes, *CCND1* and *CTSF*, demonstrated strong or

moderate, respectively, increase of expression in 24h of treatment in patient's cells (in RL cell line these genes were not expressed). Interestingly, these two genes had initially a low level of expression in the cells (**Figure 17**). The same trend was detected in MCL cell lines: mostly genes with a low expression level reacted to abexinostat (**Figure 1 and 4 in the manuscript**).

The observed variations in expression of *BCL-2* and *GSTP1* in response to the treatment in patient's cells might reflect normal fluctuations, regularly detected in experiment repeats. Only strong changes in expression, like in case of *CCND1* gene (24h) might indicate a real response of a gene to the treatment. To confirm the observed reaction of *CTSF* and *CCND1* genes to abexinostat, more samples from patients with follicular lymphoma would have to be tested.

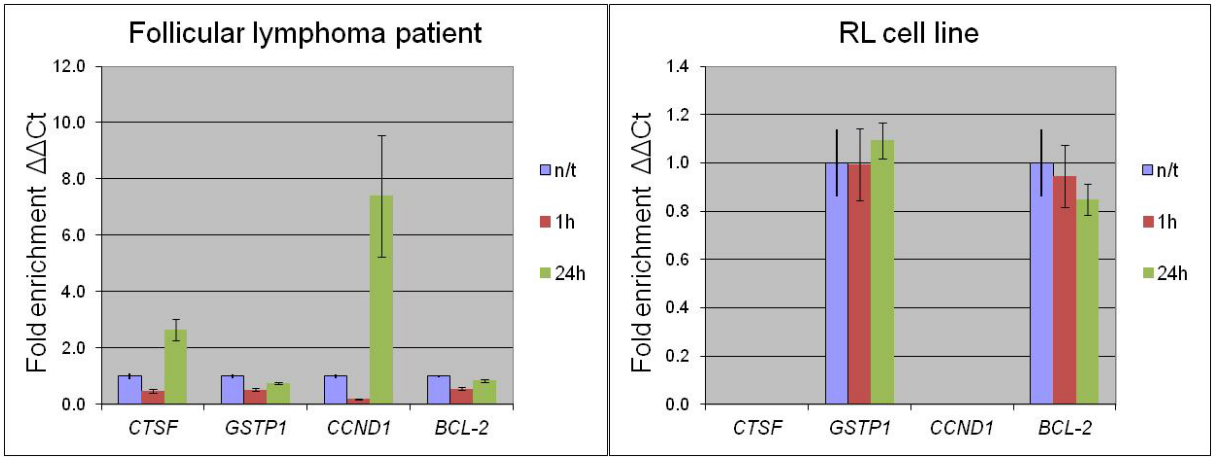


Figure 18. **Effect of abexinostat on gene expression levels in follicular lymphoma.**

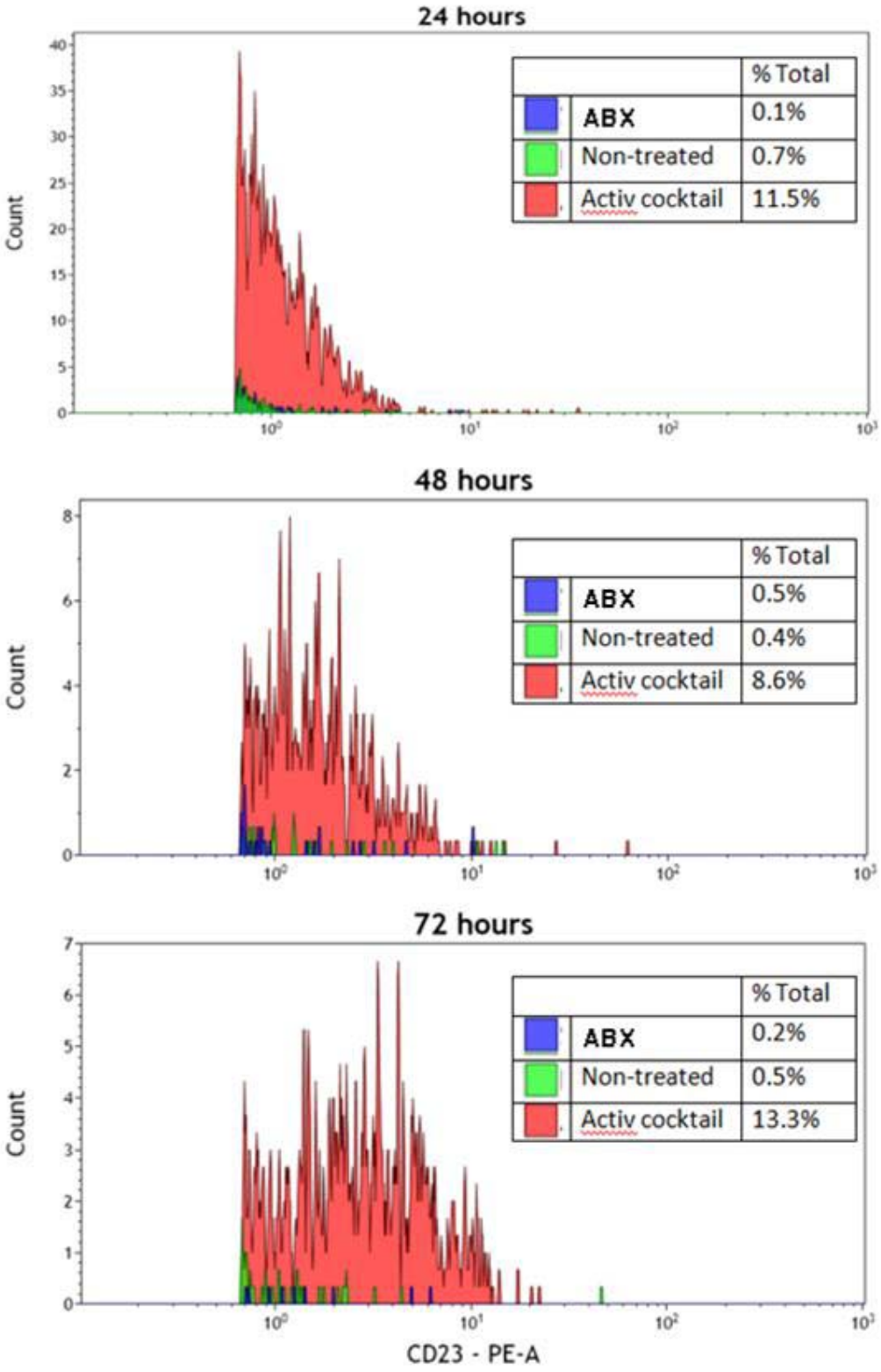
One sample from follicular lymphoma patient and RL cell line were treated with 100 nM abexinostat during 1h and 24h, then gene expression was tested using RT-qPCR. Transcript abundance was normalized to *GAPDH*, and then results from treated cell were referred to untreated cells. Three independent experiments were hold for RL cell line, the average of fold enrichment is displayed. In the patient cells, expression was analyzed in 1h and 24h in both, treated and not treated cells, then values in treated cells were related to that from untreated cells from the same time point. The values are presented as mean ± SEM.

Thus, regardless of the described downregulation of BCL-2 protein by HDACi in follicular lymphoma (Duan et al., 2005), we have not detected any decrease in *BCL-2* gene expression after abexinostat treatment. Instead, we have again observed that HDACi upregulated genes with an initially low expression level.

5. Abexinostat does not induce B-cell activation

Gene expression analysis revealed overexpression of *CD6* and *CCND1* genes in abexinostat-treated control cell lines (**Figure 4 of the manuscript**). It might indicate B cell activation triggered by the HDACi treatment. To verify this, we tested normal B cells from donors for activation using flow cytometry analysis. Marker of active B cells (CD23) and cyclin D1 were measured before and after abexinostat treatment. Activation cocktail (10 ng/ml recombinant human IL4 (Sigma), 1 µg/ml anti-human monoclonal antibodies to CD40 (G28.5 clone, Biolegend), and 20 µg/ml monoclonal anti-human IgM (clone DA4.4, kindly provided by Joelle Wiels)) was used as a positive control. Positive B cell selection was done using FITC-conjugated antibodies to CD19 marker. The experiment was repeated twice. Results are shown in **Figure 19**.

The CD23 marker was not detected in 24h, 48h or 72h upon abexinostat treatment in the normal B cells (**Figure 19A**). Neither G2/M peak of the cell cycle (**Figure 19B**), nor increase of cyclin D1 protein (**Figure 19C**) were observed in 48h or 72h of abexinostat treatment. Thus, we conclude that overexpression of *CD6* and *CCND1* genes upon abexinostat treatment in normal cells is not due to B cell activation.


24 hours
Count
% Total
ABX 0.1%
Non-treated 0.7%
Activ cocktail 11.5%
48 hours
Count
% Total
ABX 0.5%
Non-treated 0.4%
Activ cocktail 8.6%
72 hours
Count
% Total
ABX 0.2%
Non-treated 0.5%
Activ cocktail 13.3%
CD23 - PE-A

(A)

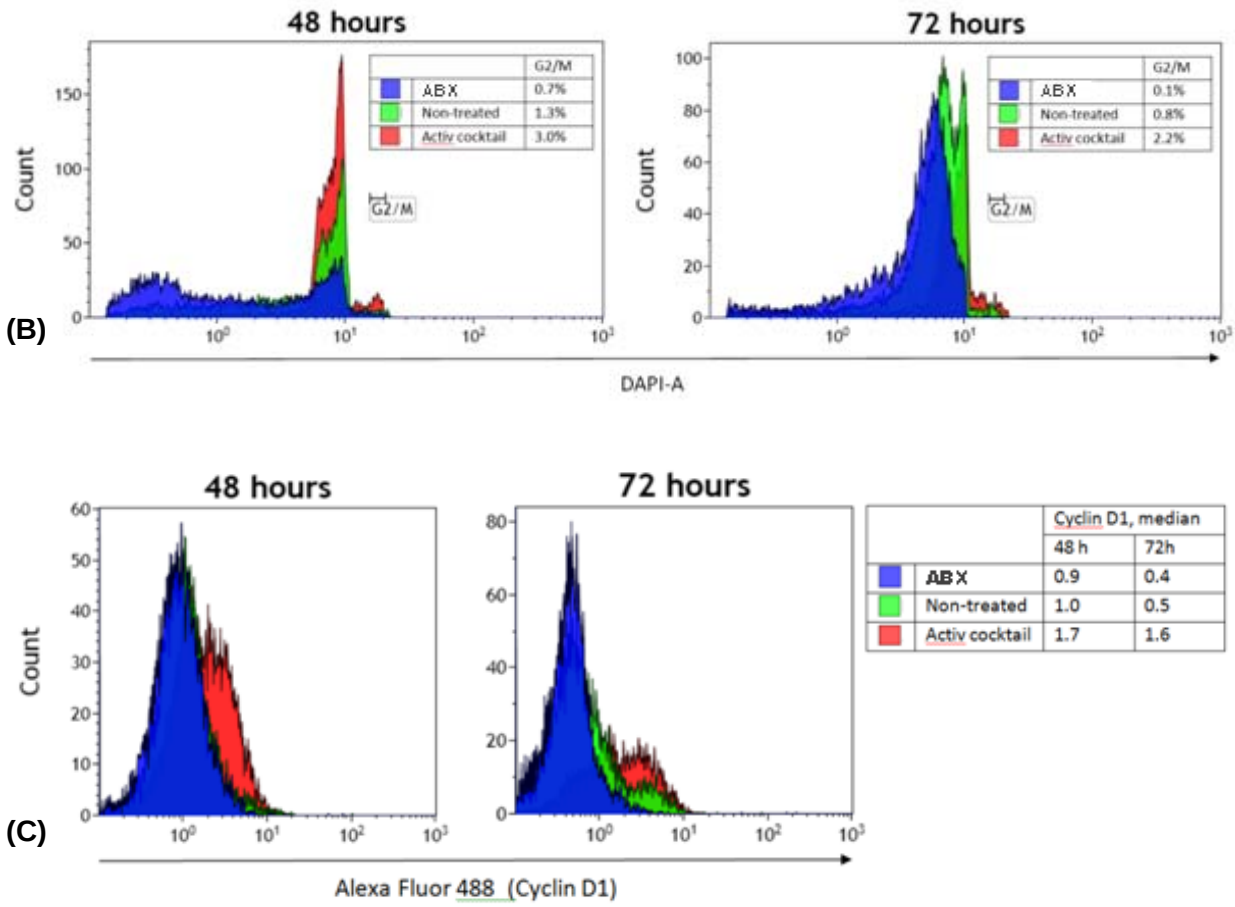


Figure 19. **Flow cytometry analysis of B-cells activation markers.**

Normal B-cells non-treated, treated with abexinostat (24h, 48h, 72h), and treated with the activation cocktail as a positive control. (**A**) CD23 (median of PE-A fluorescent intensity). (**B**) Cell cycle in normal B-cells (percentage of cells with G2/M peak). (**C**) Cyclin D1 levels (median of Alexa Flour 488 fluorescent intensity).

6. Changes in histone H3K9 modifications in gene coding sequences upon abexinostat treatment

We used chromatin immunoprecipitation followed by RT-qPCR to assess histone modifications distribution in the coding sequences of four genes in response to abexinostat treatment in MCL

cell line Granta-519 and the control cell line RPMI-8866. Two genes from the 11q13 locus, *CCND1* and *GSTP1*, one housekeeping gene *GAPDH*, and one unrelated muscle-specific transcriptional activator gene *MyoD* (inactive in lymphocytes) were tested for H3K9 acetylation and di-methylation.

In the control RPMI-8866 cell line, chromatin immunoprecipitation analysis showed pronounced reaction of H3K9 acetylation in response to the treatment in *GSTP1* gene: it increased in 1h and decreased back to the initial level in 24h (**Figure 20**). Initial level of acetylation in this gene was high in comparison with other three tested genes; this is not surprising taking into account a high activity of this gene (**Figure 21**). *CCND1*, *GAPDH* and *MyoD* genes did not reveal any marked changes in their histone H3K9 acetylation levels. In the MCL Granta-519 cell line, actively expressed genes progressively increased their acetylation: moderately (*GSTP1*, *GAPDH*) or dramatically (*CCND1*). The highest initial level of acetylation was observed in *CCND1* gene, which is the most expressed in MCL (**Figure 21**). The silent *MyoD* gene had a low level of acetylation, which progressively decreased during the treatment (**Figure 20**).

Thus, in the control cell line, inactive (*CCND1* and *MyoD1*) and house-keeping (*GAPDH*) genes had no reaction to abexinostat, whereas a highly expressed *GSTP1* (**Figure 21**) experienced hyperacetylation at 1h of treatment, but at 24h, acetylation status was normalized. In contrast, in MCL cells, active genes underwent a steady hyperacetylation, whereas the silent gene *MyoD* decreased its acetylation by half (24h).

Observed histone H3 Lys9 acetylation dynamics suggests that HDAC inhibitor causes genes hyperacetylation which is persistent in the MCL cell line, but gets balanced in normal cells in the long term. This concerns mostly active and highly expressed genes.

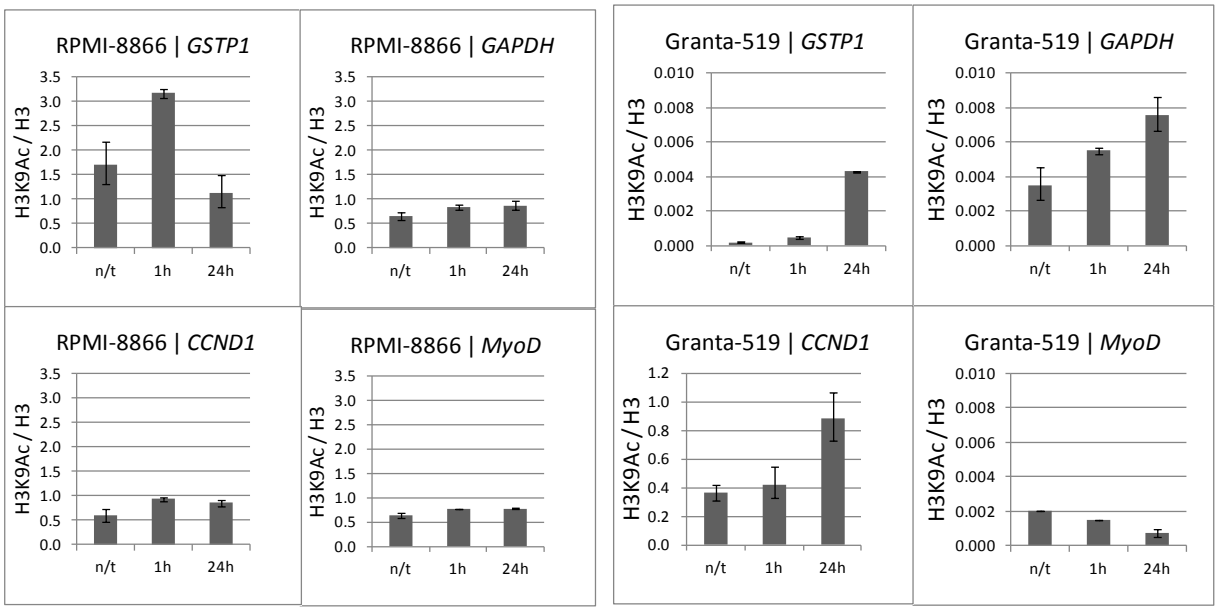


Figure 20. Histone H3K9 acetylation distribution in genes in response to abexinostat treatment in control RPMI-8866 and MCL Granta-519 cell lines.

Histone H3K9Ac distribution was measured with RT-qPCR after ChIP and normalized to total histone H3.

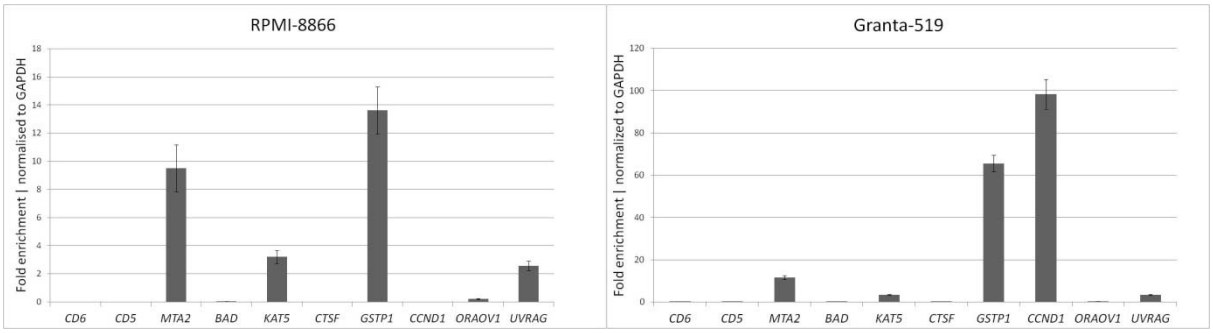


Figure 21. Gene expression in RPMI-8866 and Granta-519 cell lines.

The graphics represents average of 5 independent experiments for each cell line. 11q13 genes' expression was measured with RT-qPCR vs. *GAPDH*. The values are presented as mean±SEM.

H3K9 di-methylation showed unique a pattern in both control and MCL cell lines (**Figure 22**). The H3K9me2 levels increased at 24h of treatment: 3-4 times in the control cell line and 10-100 times in MCL. Notably, di-methylation levels in the MCL cell line initially were low as

compared to the control (0.001 *vs.* 0.5), and the observed increase in MCL was, therefore, more dramatic. This pattern might indicate presence of a common non-specific HDACi-triggered mechanism of histone hypermethylation in the coding sequences, possibly due to activation of HMTs.

Taking into account results obtained from the gene promoters (**Figure 5 in the manuscript**), we may conclude that histone H3K9 modifications in the control cell line RPMI-8866 reveal similar profiles of reaction to abexinostat both inside the genes and in the promoter regions (in the subset of sensitive to upregulation genes). However in MCL Granta-519 cell line, these profiles were partially different: in promoter zones of upregulated genes, histone acetylation and di-methylation increased strongly at 1h, and then decreased at 24h, whereas histone modification levels inside active genes (*CCND1*, *GSTP1*, *GAPDH*) were increased at 24h of treatment as compared to the controls.

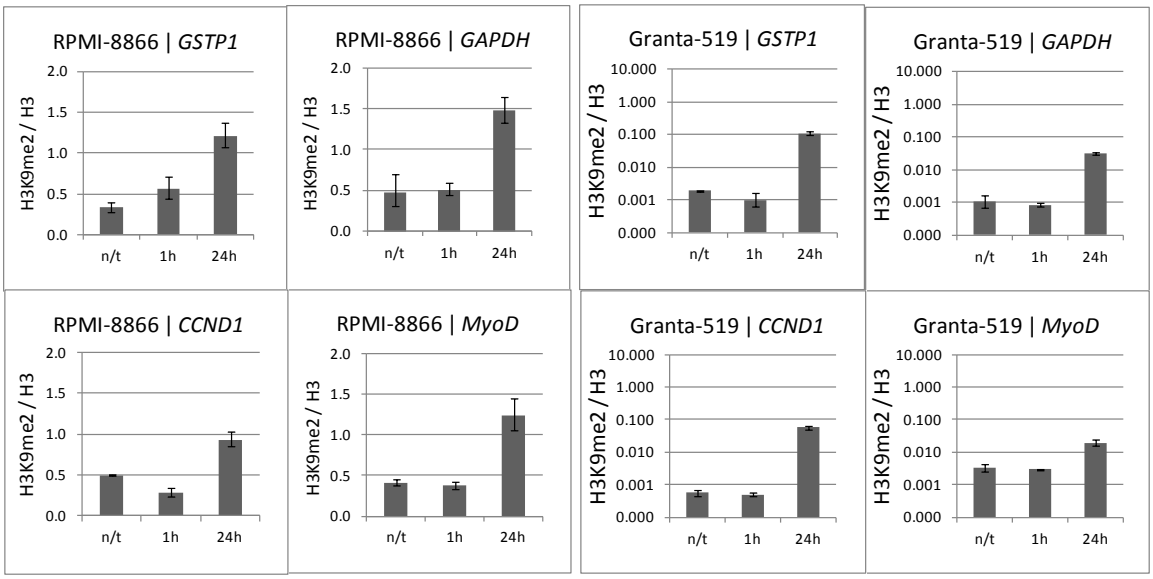


Figure 22. Histone H3K9 di-methylation distribution in genes in response to abexinostat treatment in control RPMI-8866 and MCL Granta-519 cell lines.

Histone H3K9me2 distribution was measured with RT-qPCR after ChIP and normalized to total histone H3.

II - Downregulation of cyclin D1 levels in mantle cell lymphoma

In MCL cells, HDAC inhibitors NaBu and SAHA were shown to decrease cyclin D1 protein levels without affecting *CCND1* gene expression rates (Heider et al., 2006), (Kawamata et al., 2007). Nevertheless, until now, there is no clear understanding of this phenomenon. In this part of the work, we explored potential mechanisms of cyclin D1 protein downregulation in MCL by abexinostat.

1. Abexinostat decreases cyclin D1 protein levels in MCL

To evaluate the effect of abexinostat on cyclin D1 protein levels in MCL and normal B cells, four MCL (Granta-519, Jeko-1, NCEB-1 and UPN-1) and three control (RPMI-8866, Priess and Remb1) cell lines were treated with abexinostat during 1, 24 and 48 hours. Then cells were fixed, permeabilized, stained with FITC-conjugated antibodies against cyclin D1 protein (Santa Cruz Biotechnology sc-8396) and analyzed with fluorescence-activated cell sorting (FACS) analysis. The experiment was repeated at least two times for each cell line.

In three tested MCL lines out of four, abexinostat decreased cyclin D1 protein levels as compared to non-treated cells: 1.3 times in UNP-1 (24h), 2.3 times in Jeko-1 (24h) and 1.6 times in NCEB (48h) (**Figure 23**). No changes were observed neither in Granta-519, nor in three tested control cells lines.

This data shows that abexinostat decreases cyclin D1 protein levels in MCL cells, but not in the normal B cells, though Granta-519 did not reveal intensive downregulation of cyclin D1 protein levels.

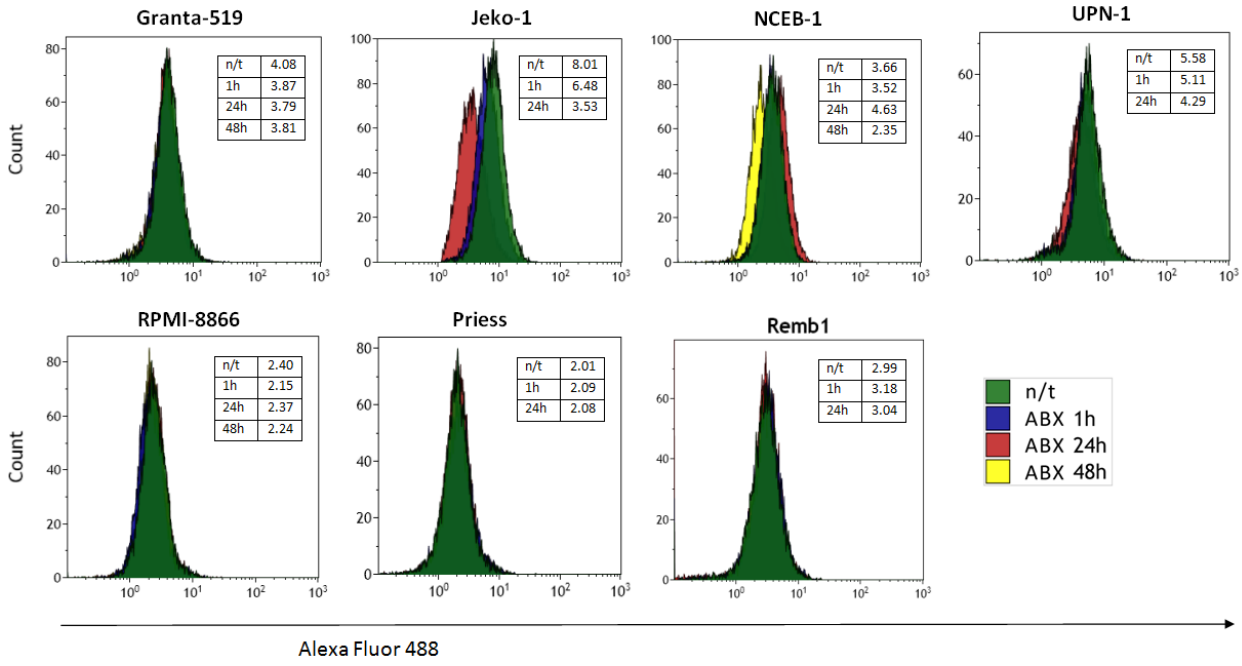


Figure 23. Representative flow cytometry histograms of cyclin D1 protein response to abexinostat treatment in the control and MCL cell lines.
The signal fluorescent intensity of Alexa Fluor 488 is given in tables as median.

2. HDACi TSA downregulates cyclin D1 protein levels

We next evaluated another HDAC inhibitor, Trichostatin A (TSA) in MCL cells to compare it with effects revealed for abexinostat. For this purpose, gene expression and cyclin D1 protein levels were assessed in Jeko-1 cell line upon TSA application. TSA was reported to be efficient in increasing general histone acetylation without growth inhibition in different types of cells while applied in concentrations 3.3, 6.6 and 33 nM (Yoshida et al., 1990). We have first tested these concentrations on naïve B cells: none of them had cytotoxic effects (data not shown). 6.6 nM was chosen as a working concentration for further analysis.

Jeko-1 cells were treated with 6.6 nM TSA for 24 and 48 hours; then fixed, permeabilized, stained with FITC-conjugated antibodies against cyclin D1 protein (Santa Cruz Biotechnology sc-8396) and analyzed with fluorescence-activated cell sorting (FACS) analysis. Cells treated with 100 nM abexinostat were used as a positive control. To analyze cell cycle populations, cells were treated with DAPI just before loading on FACS.

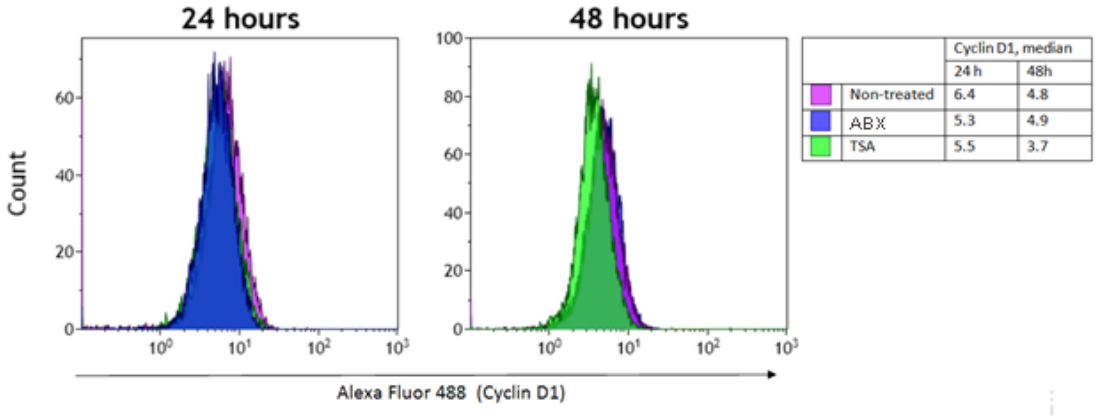


Figure 24. Effect of TSA and abexinostat on cyclin D1 protein levels in Jeko-1 cell line.

Cyclin D1 protein levels were assessed with flow cytometry using FITC-conjugated antibodies. Negative control (K-): non-treated cells; positive control (K+): cells treated with abexinostat (ABX).

At 24h, both TSA and abexinostat induced a 1.2-fold decrease of cyclin D1 protein, whereas at 48h, only TSA decreased cyclin D1 protein levels (1.3 times) (**Figure 24**). These results confirm that HDACis indeed slightly decrease the cyclin D1 levels in MCL cells.

RT-qPCR analysis did not reveal any significant changes in gene expression upon TSA treatment (**Figure 25**). Thus, the same process was observed for both abexinostat and TSA: these HDACis downregulate cyclin D1 protein without altering *CCND1* gene expression in MCL.

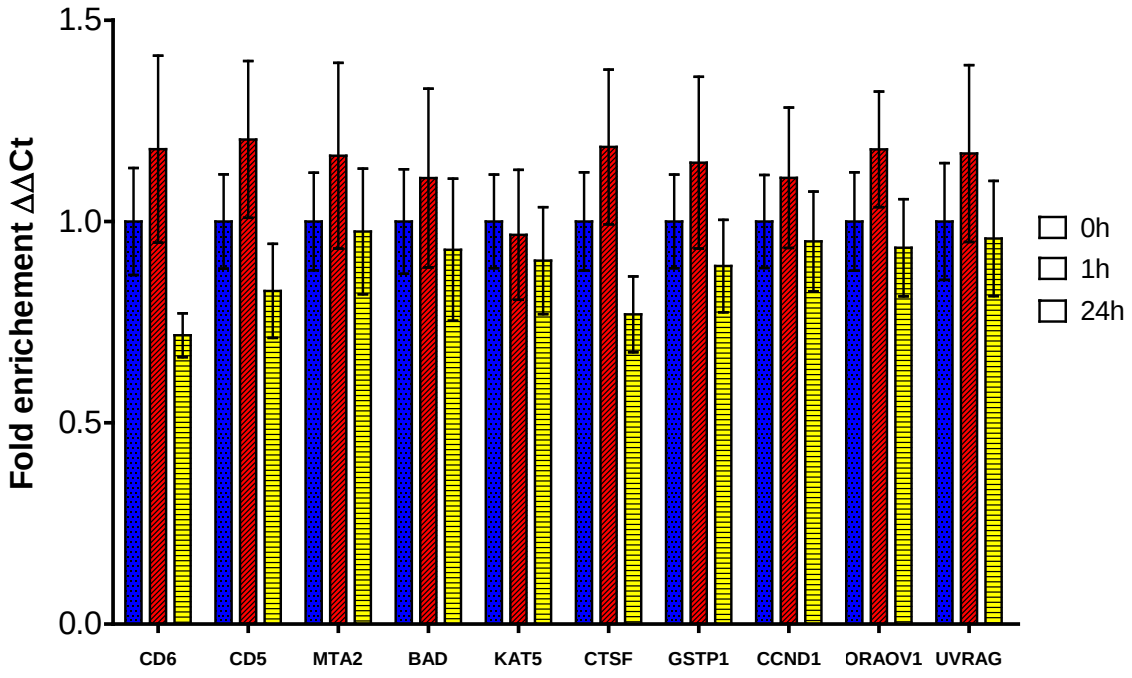


Figure 25. Expression 11q13 genes upon TSA treatment in Jeko-1 cell line.

Cells were treated with 6.6 nM TSA during 1h and 24h, then gene expression was measured using RT-qPCR vs. *GAPDH* expression. The results represent the average of three independent experiments. The data is presented as mean±SEM. Statistical test applied: 1 way ANOVA.

3. *Abexinostat triggers nucleoli disaggregation in MCL and normal B cells*

Cyclin D1 downregulation by HDACis was described by several research groups (Heider et al., 2006), (Kawamata et al., 2007), but the exact mechanism of this phenomenon has not been yet elucidated.

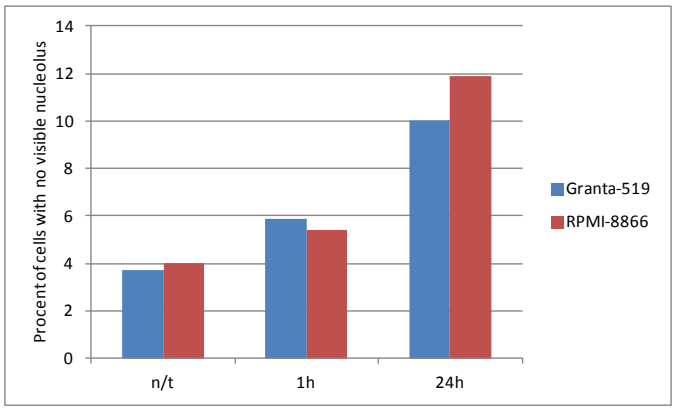
Previously we have shown that abexinostat did not reduce *CCND1* expression (**Figure 4 in the manuscript**), suggesting that gene expression is not involved in downregulation of cyclin D1 protein levels. Thus, we next analyzed the level of translation in abexinostat-treated cells.

We aimed to verify whether abexinostat would trigger any changes in nucleoli state. For this, we have used the 3D-FISH experiments in order to visualize nucleolar phosphoprotein B23, which corresponds to granular and fibrillar regions of the nucleolus. It is a structure where ribosomes are synthesized and undergo maturation for following transport to the cytoplasm where they serve for translation of mRNA to proteins.

MCL Granta-519 and control RPMI-8866 cell lines were treated with 100 nM abexinostat for 1 and 24 hours, then immunostained with anti-B23 antibody (Sigma). After 24 hours of treatment, we detected a significantly higher ratio of cells without visible nucleoli (**Figure 26**: 10% *vs.* 3% and 12% *vs.* 4% in average for Granta-519 and RPMI-8866, respectively).

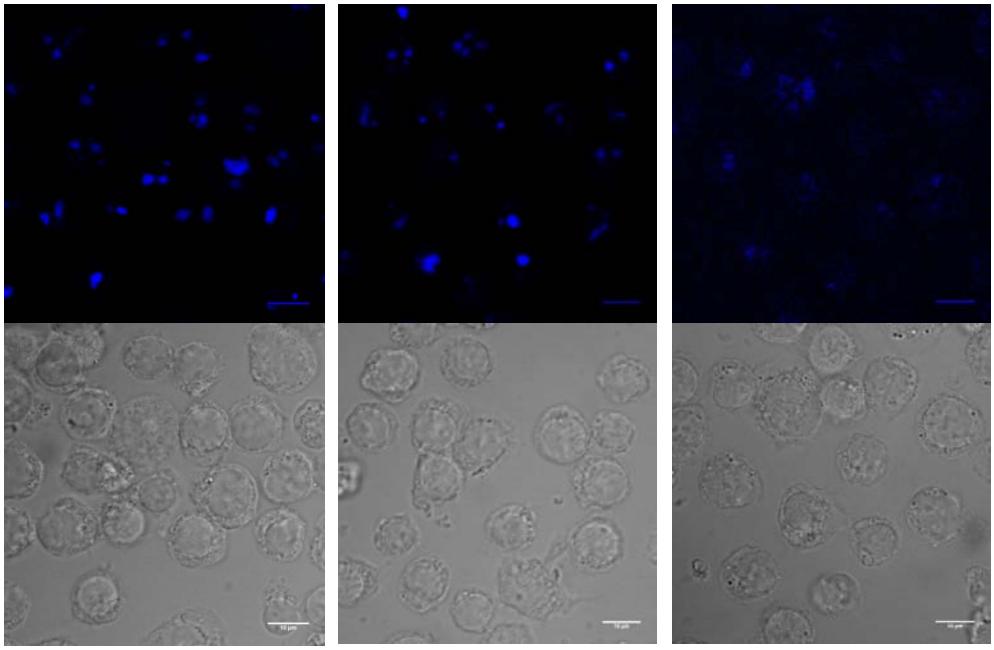
Interestingly, the simultaneous detection of nucleoli with the genes of interest (*CCND1*, *GSTP1* and *IgH*) detected a loss of their association already at 1h of abexinostat treatment (**Figure 27**). We have previously shown that *IgH*, *CCND1* and *GSTP1* genes after translocation (11;14) in MCL cells were localized in the perinucleolar space which has a transcriptionally active background (Allinne et al., 2014). Nevertheless, this dissociation after abexinostat

treatment does not lead to a decrease in the transcription level of the 11q13 genes (**in the manuscript: Figure 4** and **supplementary Figure 2**).



(**A**)

n/t 1h 24h



(**B**)

Figure 26. The number of cells with no visible nucleoli increased 24 hours after the abexinostat treatment.

RPMI-8866 and Granta-519 cells were fixed after the 1h and 24h of abexinostat treatment. Immunostaining was made to visualize nucleoli (B23 protein). (**A**) The graph shows rate of cells with reduced nucleoli at 0, 1 and 24 hours after abexinostat treatment. The dashed curve corresponds to Granta-519 cell line and continuous curve to RPMI-8866 cell line. (**B**) A confocal section of Granta-519 cell stained with antibodies against a nucleolar marker B23 (blue). n/t- not-treated; 1h and 24h - abexinostat treated. Scale bar: 5μm.

These results indicate that HDAC inhibitor abexinostat perturbs nucleoli structure; this might have effect on ribosomal level and thus might affect protein translation process. Therefore, we further tested main ribosomal rRNA's levels upon abexinostat treatment.

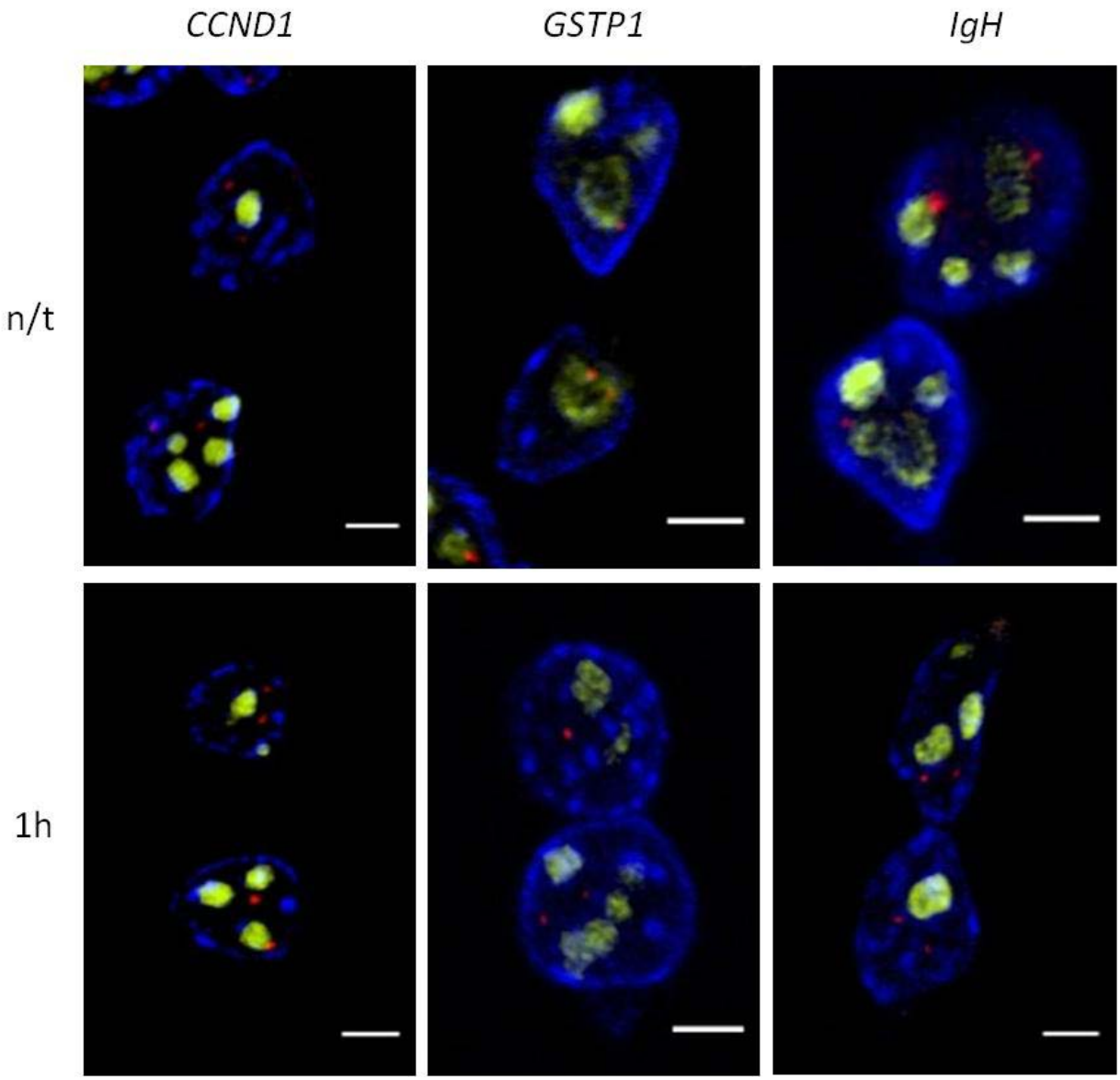


Figure 27. Genes lose their association with nucleoli after abexinostat treatment.

Fluorescent *in situ* hybridization was performed to reveal *CCND1*, *GSTP1* and *IgH* genes (red), then immunostaining was used to visualize heterochromatin (H3K9me3, blue) and nucleoli (B23, green). Cell line presented: Granta-519. n/t – without treatment; 1h – abexinostat treatment. Scale bar: 5um.

4. Abexinostat does not affect rRNA levels

To further investigate abexinostat effect on nucleoli, we assessed rRNA quantity in five MCL and five control cell lines before and after treatment. Cells were treated with 100 nM abexinostat during 1 and 24 hours, total RNA was extracted, and a relative quantity of 18S + 28S rRNAs to total RNA was measured using Bioanalyzer (Agilent Technologies). For each cell line, from 2 to 6 repeats of the experiment were performed.

No significant changes in rRNA levels were detected upon abexinostat treatment in both MCL and control cell lines (**Figure 28**).

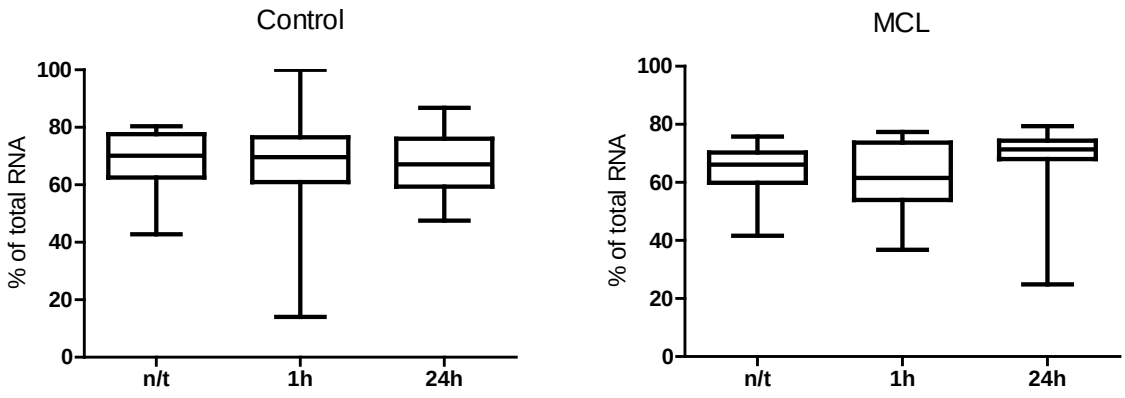


Figure 28. **Dynamics of rRNA content in abexinostat treated cells.**

5 MCL and 5 control cell lines were treated with abexinostat during 1 and 24 hours. Total RNA was extracted from treated and non-treated cells. Relative level of 18S + 28S rRNA to total RNA was measured using Bioanalyzer, Agilent Technologies, Inc. The box represents the first and third quartiles, the band inside the box is the median, the ends of the whiskers represent the minimum and the maximum of the total data.

5. MG132 proteasome inhibitor decreases cyclin D1 protein levels

As cyclin D1 protein reduction by abexinostat was not caused by chromatin modifications, gene expression changes or ribosomal state, we hypothesized that HDACi might affect cyclin D1 on the level of its degradation. Cyclin D1 turnover is regulated by 26S proteasome (Alao, 2007), thereby its inhibition would be expected to increase cyclin D1 protein level.

We treated Jeko-1 cell line with proteasome inhibitor MG132 (25 mM) during 24h or 48h and tested abexinostat effect on cyclin D1 protein levels using flow cytometry and FITC-conjugated antibodies against cyclin D1 (Santa Cruz Biotechnology sc-8396). Surprisingly, we observed cyclin D1 protein reduction upon MG132, applied alone or together with abexinostat (respectively 1.26 and 1.45 times in 24h; 1.38 and 1.35 in 48h) (**Figure 29**).

Remarkably, MG132 induced G_1 arrest: treated cells accumulated in G0/G1 phase of the cell cycle (24h and 48h). Cells treated with only abexinostat revealed partially this effect: G2/M

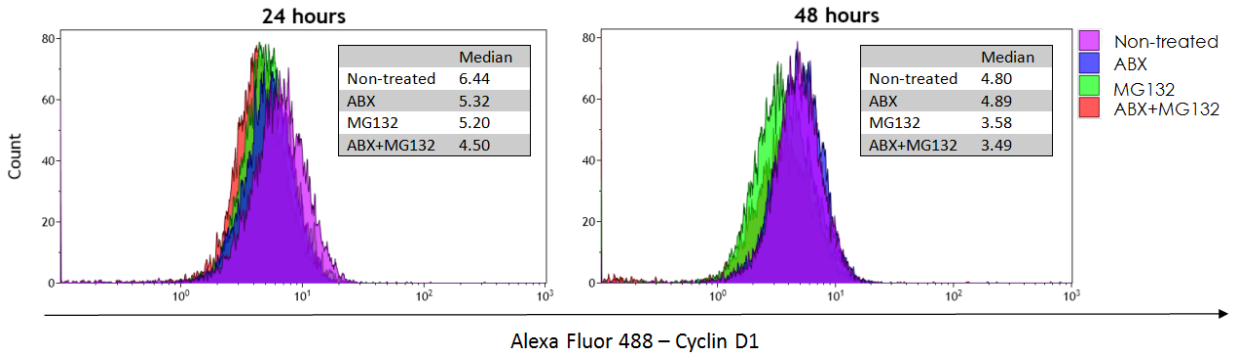


Figure 29. Effect of proteasome inhibitor MG-132 on Cyclin D1 protein level in Jeko-1 cell line.

Jeko-1 cells were treated with 25 mM MG132 during 24h or 48h. Cyclin D1 protein levels were measured with FACS using FITC-conjugated antibodies against cyclin D1. Median of cyclin D1 fluorescent intensity (Alexa Fluor 488) is shown in tables.

peak of the cell cycle decreased in 48h. Moreover, MG132 triggered cells apoptosis after 48h of treatment, which was not observed in the cells treated only with abexinostat (**Figure 30**).

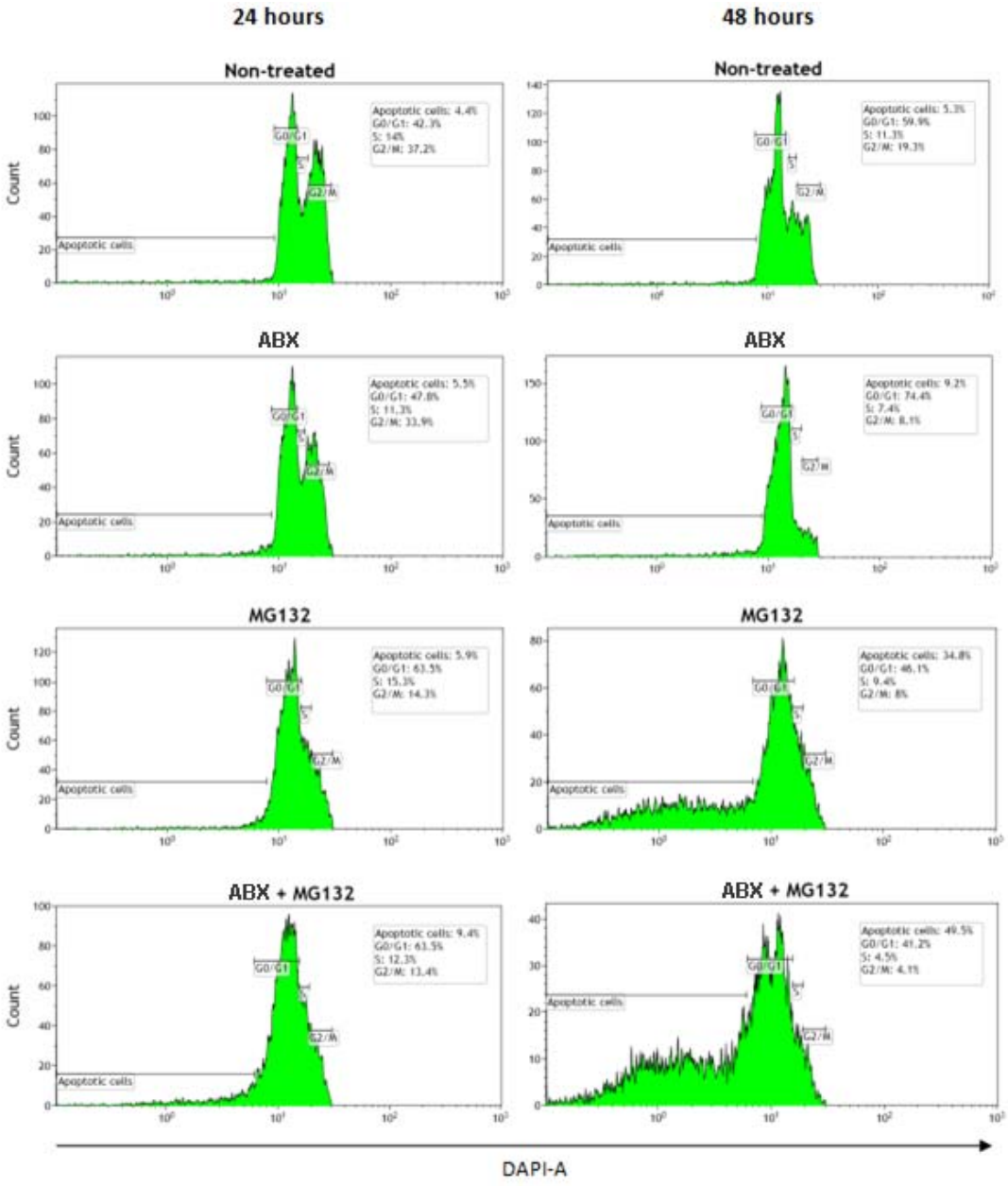


Figure 30. Effects of abexinostat and MG132 on cell cycle progression of Jeko-1 cell line.

Jeko-1 cells were treated with proteasome inhibitor MG132 (25 mM) and abexinostat (100nM) for 24h or 48h. Cells cycle and apoptosis level were assessed with DAPI.

III – Epigenetic state of rearranged loci in MCL and normal primary cells

Usage of established cells lines is very practical due to their ability to indefinitely proliferate, whereas primary cells extracted directly from donors have very limited lifespan. Nevertheless, there are advantages of using primary cells over cell line, because the latter is transformed and has a deviation from normal primary cells. That is why we wanted to assess chromatin state and gene expression in the samples from patients with MCL and healthy donors. Thus, in this part of the work, we evaluated 11q13 gene expression, chromatin state and abexinostat effect in one sample from MCL patient and in normal B cells from healthy donors.

1. *11q13 genes are overexpressed in MCL patient in the same way as in MCL cell lines.*

One sample from a patient with MCL and three samples from healthy individuals were analyzed for gene expression near the (11;14) translocation point. Six genes out of ten were overexpressed in a patient with MCL as compared to normal B-lymphocytes: *CD6*, *CD5*, *CTSF*, *GSTP1*, *CCND1*, *ORAOV1* (**Figure 31**). The same pattern was observed in MCL cell lines (**Figure 1 in the manuscript**). Thus, cell lines and primary cells from humans have the same background of gene overexpression after the t(11;14) translocation.

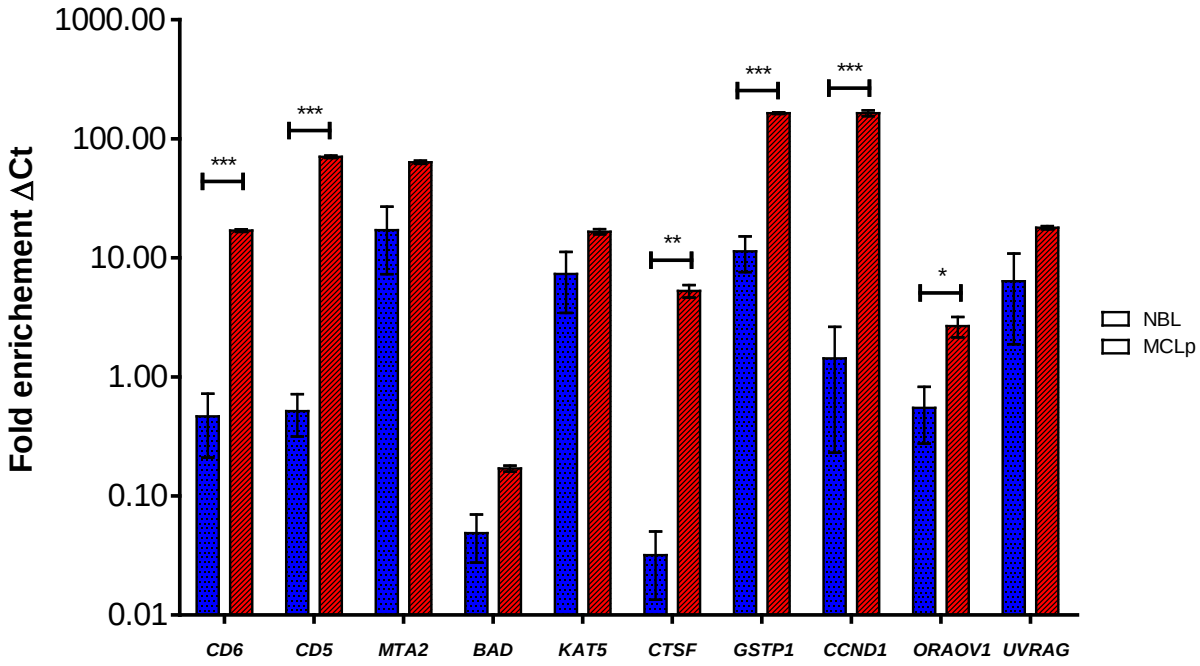


Figure 31. Gene expression levels of selected 11q13 genes around the (11;14) translocation point in human samples.

The data represents the average of genes expression in normal B-lymphocytes (NBL) obtained from three healthy individuals and in MCL cells from one patient (MCLp). Transcript abundance is normalized to *GAPDH*, and presented on a base 10 logarithmic scale. The value 1 corresponds to *GAPDH* expression. The data is presented as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001 (unpaired Student's *t* test relative to control).

2. Abexinostat downregulates 11q13 genes' expression in MCL patients' cells but not in the normal B cells

Next, we have evaluated two samples of normal B cells from healthy individuals and one sample from the MCL patient for 11q13 gene expression in response to abexinostat treatment. As long as the cells were not immortalized in the culture and their survival was decreasing in

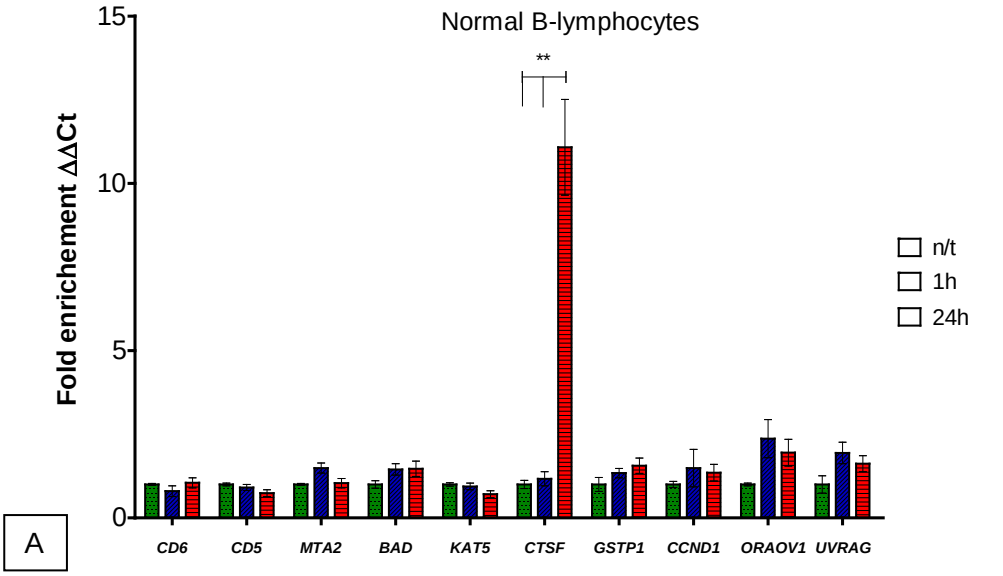
time, gene expression was assessed at 1 and 24 hours both in treated and untreated cells. Then expression in treated cells was related to untreated ones from the same time point.

In normal B cells, only *CTSF* gene reacted to the abexinostat treatment with an 11-fold increase of expression at 24h (**Figure 32A**). In case of MCL cells from the patient, all ten tested genes had a tendency to decrease their expression at 1h and then increase it at 24h (**Figure 32B**).

Thus, normal B-lymphocytes from humans revealed a profile of gene expression similar to normal lymphoblastoid cell lines in reaction to abexinostat: one gene in NBL (*CTSF*) and four genes in LCL (*CTSF*, *CD6*, *GSTP1 and CCND1*) increased their expression at 24h of treatment. Interestingly, *CTSF* was the gene with the lowest expression in NBL.

MCL cells from the patient showed a different pattern of gene expression as compared to MCL cell lines. In the latter, four genes (*CD6*, *CD5*, *CTSF*, *GSTP1*) increased their expression at 24h of abexinostat treatment, while in the MCL patient, genes showed a quick decrease (1h) in expression with a gradual increase at 24h.

Here, we observe abexinostat-triggered downregulation of *KAT5*, *CTSF*, *CCND1* and *UVRAG* genes expression in cells from one MCL patient. More samples from MCL patients should be analyzed to confirm this pattern.



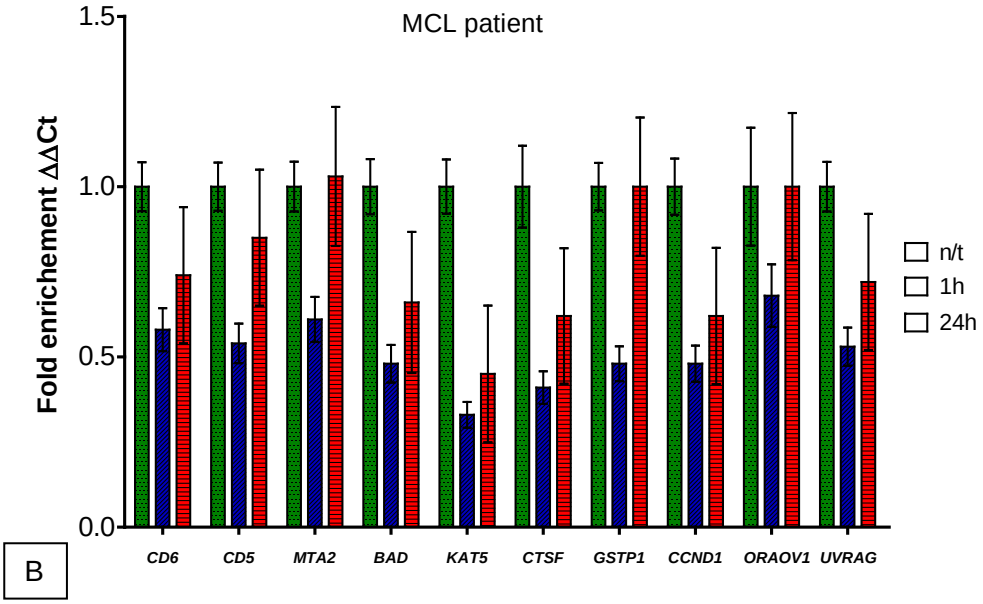


Figure 32. Effect of abexinostat on 11q13 genes' expression levels.

The cells were treated with 100 nM abexinostat, and then 11q13 genes' expression levels were measured by RT-qPCR vs. *GAPDH*. Expression was analyzed in both treated and non-treated cells, in 1h and 24h, then expression in treated cells was related to that from untreated cells of the same time point. The values are presented as mean ± SEM. (**A**) Normal B-lymphocytes from healthy individuals. The values represent an average of two samples of normal B-lymphocytes. **p<0.01 (1 way ANOVA with Turkey post-test). (**B**) MCL cells from a patient. The values represent a mean of technical triplicates from one patient's sample.

3. *Effect of abexinostat treatment on histone H3K9 modifications in gene coding sequences*

We next used chromatin immunoprecipitation followed by RT-qPCR to assess histone modification distributions (H3K9Ac, H3K9me2, H3K9me3) within the coding sequences of 4 genes (*CCND1*, *GSTP1*, *GAPDH*, *MyoD*) in response to abexinostat treatment in the MCL cells from patient.

Analysis of H3K9me3, a mark of constitutive heterochromatin, revealed a two-fold decrease of H3K9me3 level in all four tested genes at 24h of treatment (**Figure 33**). This corresponds to the results of immunohistochemistry analysis where abexinostat was shown to trigger global heterochromatin desegregation in 24h of treatment (**Figure 3 in the manuscript**). At 1h, the H3K9Me3 levels varied depending on the gene: in *GSTP1* tri-methylation dramatically decreased, in other three genes, it either did not change (*MyoD*) or moderately increased (*GAPDH*, *CCND1*).

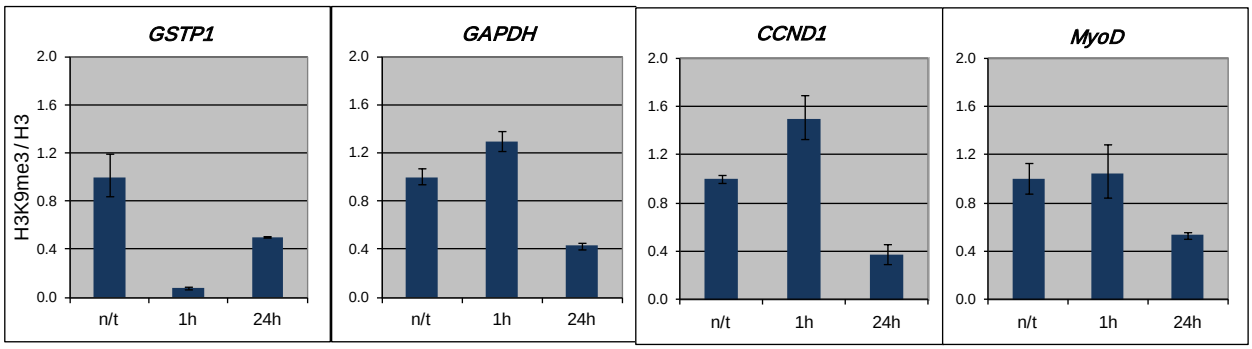


Figure 33. **Distribution of histone H3K9me3 marks in response to abexinostat treatment in MCL cells from the patient.** Data is normalized to total histone H3.

In response to abexinostat treatment, H3K9 acetylation in three active genes (*GSTP1*, *GAPDH*, *CCND1*) increased immediately (1h) (**Figure 34A**). The most dramatic increase was

observed in the *GSTP1* gene (8-fold). At 24 hours, acetylation levels were substantially reduced in *GSTP1* and *GAPDH*, whereas in *CCND1* acetylation remained increased twofold. The silent gene *MyoD* did not reveal any changes. A similar pattern was observed in the control RPMI-8866 cell line, whereas all MCL cell lines demonstrated an increase of H3K9 acetylation (**Figure 20**). Thus, cells from MCL patient revealed a reaction similar to normal cells, but with more pronounced changes in expression and with overexpression of *CCND1* at 24h.

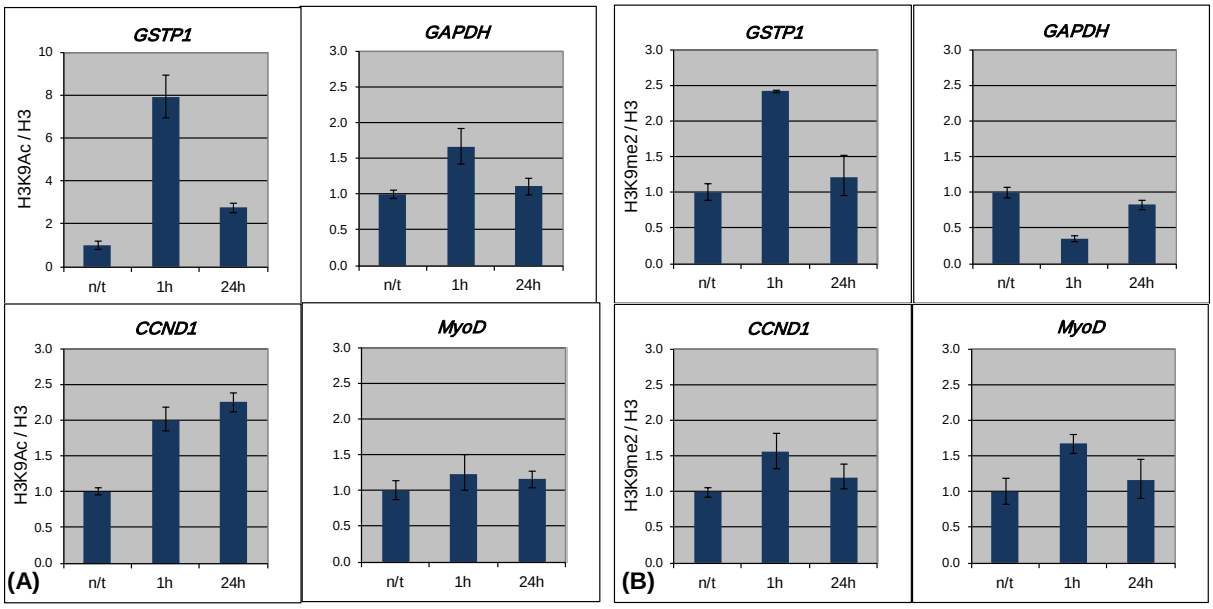


Figure 34. Histone modification distributions in the genes in response to abexinostat treatment in MCL patient.

H3K9Ac (**A**) and H3K9me2 (**B**) distributions were measured with RT-qPCR after ChIP and normalized to total histone H3.

H3K9me2 levels showed a quick response to abexinostat treatment: and increase (*GSTP1*, *CCND1*, *MyoD*) or decrease (*GAPDH*) of H3K9me2 levels, whereas at 24h, all levels returned to the initial values (**Figure 34B**). This substantially differs from the results obtained on the cell

lines, where both control and MCL, showed increase of H3K9me2 levels in 24 of treatment (**Figure 22**).

In summary, MCL cells from the patient revealed a different response to abexinostat treatment at the level of histone modifications as compared to MCL cell lines. The same was observed in the gene expression analysis (**Figure 32B** and **Figure 4 in the manuscript**). That might indicate a substantially different cellular background in intact human cells and established cell lines. To verify that, more samples from MCL patients have to be analyzed.

IV - CONCLUSIONS

This work gives a new insight on the mechanisms of gene expression regulation in lymphomas. Lymphomas are the types of cancer where almost 100% of cases are caused by chromosomal translocations triggering aberrant gene expression. The most common consequences of chromosomal translocations are: gain or loss of genetic information and formation of fusion genes bearing often oncogenic qualities. However, some translocations do not lead to any of these structural abnormalities, but still trigger the malignant transformation by the mechanisms which are not clearly understood. In this work, we have studied mechanisms of gene expression misregulation after chromosomal translocation on an example of mantle cell lymphoma.

MCL harbors a (11;14)(q13;q32) translocation, which leads to cyclin D1 proto-oncogene overexpression. Previous studies by our and other research groups hinted at epigenetic mechanisms involved in this upregulation (Allinne et al., 2014), (Bennaceur-Griscelli et al., 2004). Therefore, we have concentrated on studying epigenetic processes that might be involved in MCL development. We have studied chromatin state and 11q13 genes' expression, and elucidated effects of epigenetic drugs (HDAC inhibitors) in MCL and control cells.

In the first part of the work, we used ChIP-on-chip analysis to demonstrate an active epigenetic background in both the rearranged loci (11q13 and 14q32) after the translocation t(11;14) in MCL. Analysis of gene expression arrays from GEO database and RT-qPCR gene expression

analysis revealed overexpression of a group of genes (including *CCND1* and *GSTP1*) in MCL as compared to normal cells. These overexpressed genes are spread along 15 Mb region, and notably, they are located on both sides from the chromosome breakpoint: i.e. in the locus which is relocated to der14 and on the der11 which is not replaced.

ChIP-on-chip array have uncovered that these upregulated genes had markedly different histone H3 Lys9 modifications signature as compared to the rest of the genome. Intriguingly, this signature was different even in the control cells, thus before the translocation occurs.

As soon as it became evident that epigenetic processes are involved in genes' upregulation in MCL, it was interesting to evaluate epigenetic drugs influence on gene expression and chromatin state to understand better the underlying mechanisms of misregulation in lymphomas. We have tested HDAC inhibitors, a new class of anti-cancer drugs, shown promising results in lymphomas cure, though its exact mechanism of action is far from being clear.

The direct consequence of HDACis application is an increase of histone acetylation levels in genome. Indeed, immunostaining with antibodies against constitutive mark of heterochromatin (H3K9me3) demonstrated global chromatin activation upon Abexinostat treatment: in 24h in both MCL and control cells, the amount of heterochromatin dramatically decreased. Nevertheless, ChIP-on-chip analysis did not show any global changes in acetylation status of genes' promoters (in MCL and control cells). Instead, only a small proportion of genes revealed a response to the drug: the genes sensitive to upregulation by the translocation (11;14) (referred as '*upregulated*'). Moreover, acetylation in this subset of genes behaved in different ways depending on the cell line: decrease or increase of Ac levels in 1h, and then decrease in 24h. Notably, H3K9me2 marks as well intensively reacted to abexinostat treatment, but

similarly to acetylation, only in the small subset of upregulated genes. Thus, only small proportion of genes reacted to the HDACi treatment, whereas the rest of genes were sheltered from its influence; and observed changes were triggered by HDACi rather indirectly.

Then, we tested abexinostat influence on 11q13 genes' expression. RT-qPCR has shown that not all genes sensitive to upregulation by the translocation (11;14) reacted to abexinostat treatment. Mostly genes with initially low expression (lower that *GAPDH*) increased their expression in 24h of treatment. For example, abexinostat increased *CCND1* expression levels in MCL cells, but not in the control. The same scenario was observed in follicular lymphoma, which has (11;18) translocation leading to *BCL-2* overexpression. Abexinostat did not change *BCL-2* expression levels, but increased those of *CCND1* and *CTSF*, which were initially very less expressed in the follicular lymphoma cells.

A number of findings demonstrated HDACi-induced downregulation of cyclin D1 protein (Heider et al., 2006), (Kawamata et al., 2007). However mechanisms of this process were not understood. In the second part of the work, we have investigated HDACis impact on the cyclin D1 levels.

Indeed, FACS analysis showed that HDACis abexinostat and TSA decreased amount of cyclin D1 protein in MCL cell lines in 24h of treatment. However, efficiency of cyclin D1 degradation depended on the cell line type. Considering that our results had shown these HDACis to not alter *CCND1* expression in MCL, we searched for possible mechanisms of cyclin D1 protein downregulation on other levels.

We have assessed nucleoli state using 3D-FISH and detected a significantly higher ratio of cells with dramatically reduced nucleoli in 24h of treatment (in both MCL and control).

However, 18S + 28S rRNA levels analysis did not reveal any changes upon abexinostat treatment. Then, we have verified whether HDACi might impact on cyclin D1 degradation. We have inhibited proteasomes in MCL cells and evaluated cyclin D1 protein levels using FACS. Paradoxically, proteasome inhibitor MG132 as well led to reduction of cyclin D1 protein amount. Thus, apparently abexinostat affects neither translation process on the ribosomal level, nor cyclin D1 protein degradation by proteasome.

In the third part of the work, we have verified whether abexinostat would have the same effects in MCL primary cells as those which have been discovered in MCL cell lines. For this, we have tested gene expression and chromatin state in one sample from patient with MCL and compared it to normal B-lymphocytes (NBL) extracted from blood of donors (three samples).

First of all, we have noticed that 11q13 genes are overexpressed in MCL patient as compared to NBL in the same way as in MCL vs. control cell lines. Then, we have applied abexinostat and found out that in NBL it triggered the same type of changes as in the normal cell lines: one gene with lowest initial expression substantially increased it in 24h of treatment. However, in the MCL patient's cells, genes showed a totally different response to abexinostat: some part of the genes decreased its expression already in 1h of the drug application, including *CCND1* gene. Apart from that, histone modifications inside the genes demonstrated as well other type of response to the treatment in contrast to MCL cell lines. To verify observed differences, more MCL patient's samples have to be analyzed. Nevertheless, taking into account the fact that primary cells differ from cells established in the culture, it is possible to assume that we witnessed different impact of abexinostat on the cellular processes due to deviation of biological background in cell lines and in primary cells.

DISCUSSION

I – Epigenetic mechanism of gene upregulation in MCL

All chromosomes have their own location in the nucleus according to their level of activity in the different types of cells (Bolzer et al., 2005). Genes' and genomic regions' positions can be changed depending on the cell type, the cell cycle stage and in case of pathologies (Bartova and Kozubek, 2006). The fact that genomic territories are evolutionary conserved suggests its important role in genomic processes. Indeed, correlation between genomic sequence position and its expression level was demonstrated (Takizawa et al., 2008).

In this context, translocation (11;14)(q13;q32) in mantle cell lymphoma might provoke global changes in genes' transcription activity due to repositioning to a new nucleolar surroundings. 11q13 and 14q32 loci are located in the different nucleolar regions and have different levels of gene activity. For instance, *CCND1* gene is not expressed in normal B cells, in contrast, immunoglobulin *IgH* genes are abundantly expressed. In B-lymphocytes, chromosome 14 is situated in the nucleolar center and is associated with nucleolus due to actively transcribed rRNA genes situated in this chromosome. Chromosome 11, in opposite, is found near nucleolar periphery. Previously, it has been demonstrated by our research group that one allele of 11q13 locus translocates in the space and takes more central position on the derivate of chromosome 14 in MCL (Allinne et al., 2014) (**Figure 35A**). Thus, the 11q13 locus together with the situated on it *CCND1* gene, replaces into the active nucleolus region revealing high

transcription levels. Interesting, derivate of chromosome 11 (which contains *GSTP1* gene) does not move from the nucleus periphery, however it acquires position close to nucleolus surface (**Figure 35B-C**; data not published).

Relocalization to the new transcriptionally active surroundings in the nucleus most likely would impact the gene transcription activity. Indeed, we have demonstrated that apart from *CCND1* and previously reported *GSTP1* (Bennaceur-Griscelli et al., 2004) genes, a big group of genes is overexpressed after (11;14) translocation in MCL. Analysis of gene expression arrays from GEO database revealed 36 genes spread on 15 Mb region on both sides from the breakpoint on the chromosome 11. Using RT-qPCR we tested some genes from this list and proved that their expression is higher in MCL *vs.* control B cells. We observed the same picture both in cell lines and in primary cells from humans.

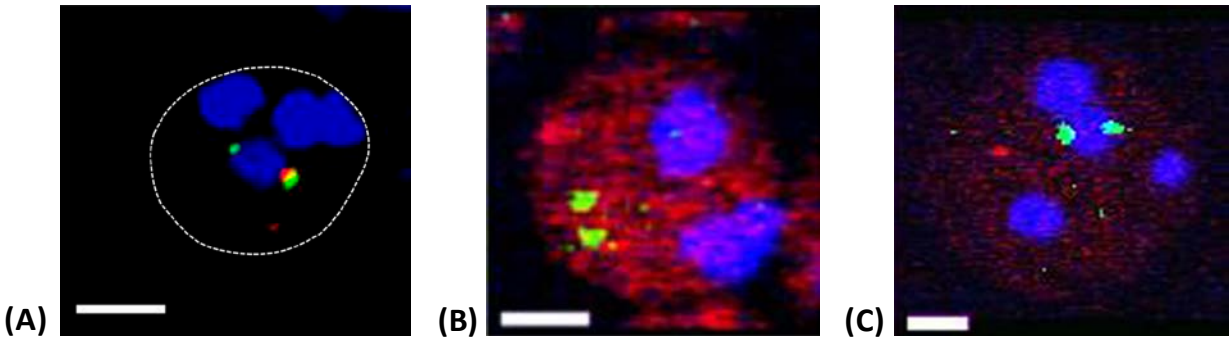


Figure 35. Nucleolar localization of the *CCND1* alleles, der14 IgH and der 11 *GSTP1* in MCL and normal B cells.

(**A**) Perinucleolar relocalization of the der14 IgH and *CCND1* alleles in the MCL Granta-519 cell line. A representative image of double-labeled DNA FISH. *IgH* is green, *CCND1* is red, and nucleolus (B23) is blue. Scale bar represents 5μm. Image is from (Allinne et al., 2014). (**B-C**) A confocal section of NBL (B) and Granta-519 MCL cells (C) stained with antibodies against B23 (a nucleolar marker, blue) and hybridized in situ with the *GSTP* BAC probe (green). Scale bar: 5um. After the translocation, the position of the *GSTP* gene region vs. the nuclear periphery did not change significantly, while its proximity to the nucleolus has greatly increased (0,65 um in Granta-519 cells vs. 1,37 um in NBL). The difference in the localization of the *GSTP* locus vs. the surface of the nucleolus is statistically significant ($p < 10\text{-}15$).

Revealed data calls into question the previous model explaining *CCND1* overexpression in MCL, where a strong *IgH* Eμ enhancer was hypothesized to upregulate *CCND1* transcription. First of all, single *IgH* enhancer misregulating genes on over 15 Mb region seems being very unlikely. Secondly, experiments on transgenic mice demonstrated that mere expression of the *CCND1* gene under the control of the *IgH* Eμ enhancer is not sufficient to develop lymphomas (Fiancette et al., 2010). Finally, in our previous work a colocalization of *IgH* and translocated *CCND1* loci with nucleolus-derived factor, nucleolin, was discovered, which presumably activates transcription (Allinne et al., 2014).

Indeed, our study of epigenetic landscape in the rearranged loci revealed more active background in the translocated 11q13 locus (higher level of H3K9Ac marks in MCL *vs.* control). Interesting, 14q32 locus as well demonstrated more active background after the translocation. But this should not come as a surprise as replacement of a big part of chromosome cannot happen without affecting neighborhood, including its epigenetic level.

Analysis of histone modification marks in genes' promoters has shown the 11q13 locus to have distinct epigenetic signature. In the control cell line, its H3K9Ac levels are almost twice lower than in the rest of genome, whereas after the (11;14) translocation acetylation levels increases twice and become the highest over the rest of genome. Histone methylation levels are as well affected by the translocation. Normal B cells have low H3K9me2 levels in the 11q13 locus, which are changed after the translocation in a cell line-dependent way. For example, in Jeko-1 and UPN-1 H3K9me2 levels increase twice (as compared to the control), but in Granta-519 it substantially decreases in the entire chromosome 11.

In summary, the findings presented here offer another mechanism of *CCND1* and other 11q13 genes overexpression in MCL. It favors large-scale epigenetic changes to be responsible for

genes' upregulation. It is reasonable to expect that chromosomal translocations in other types of lymphomas may provoke similar mechanisms of misregulation.

II – Are the 11q13 genes prone for being upregulated ?

In the current work, we have discovered that subset of genes which are overexpressed after the (11;14) translocation (*upregulated*) have distinct epigenetic signature and their histone acetylation levels are very high. From **Figure 36**, it is obvious that ratio of H3K9Ac/H3K9me2 levels is markedly higher in the subset of *upregulated* genes than in the rest of genome and even than in the 11q13 locus.

Such active epigenetic signature in the subset of *upregulated* genes in Jeko-1 and UPN-1 cell lines or in the entire 11q13 locus in Granta-519 can simply reflect the fact of these genes being upregulated after the translocation. Indeed, the 11q13 locus changes its normal positioning and relocalizes to the new active chromatin surroundings, what obviously influences on the transcriptional rates. However, active epigenetic signature in the subset of *upregulated* genes exists as well in the control cell line, thus yet before the translocation occurs. This might indicate the possibility that these genes can be prone for upregulation due to their more active epigenetic background.

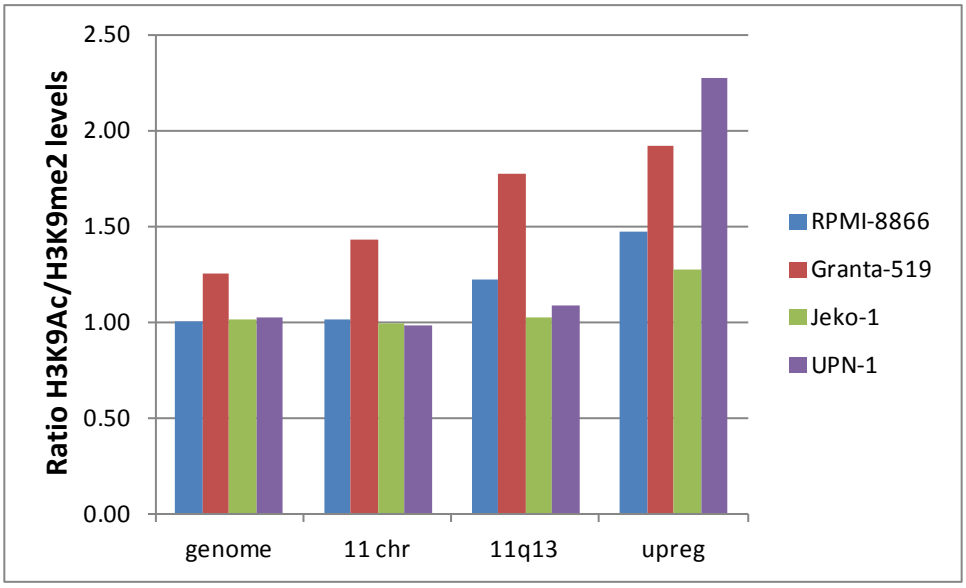


Figure 36. Ratio H3K9Ac/H3K9me2 levels in gene promoters in MCL and control cell lines.

Chromatin from control cell line (RPMI-8866) and three MCL cell lines (Granta-519, Jeko-1, UPN-1) was immunoprecipitated with antibodies against H3K9Ac, H3K9me2 and panH3 as a reference. Enrichment in acetylation and methylation normalized to panH3 was assessed using Agilent Human Promoter Microarray. Statistically significant H3K9Ac and H3K9me2 peaks were calculated in the entire genome, the 11 chromosome, the 11q13 locus, and in the genes sensitive to upregulation after the translocation t(11;14) ('*upregulated*'). H3K9 acetylation and H3K9 methylation levels (amount of statistically significant histone modification peaks divided by number of genes in the region analyzed) were calculated, and then ratio of H3K9Ac/H3K9me2 was taken, which is represented on the chart.

Important marks such as H3K9Ac, H3K14Ac, H3K4me3 and others are highly correlated with active promoters (Jenuwein and Allis, 2001). Enrichment in this histone marks near transcription start sites (TSS) facilitates binding of transcriptional machineries. However, their mere presence at the promoter regions does not initiate transcription, as far as other components including RNA polymerase II and associated transcription factors have to be attracted to the promoter and be initiated. This can explain why *upregulated* genes with high

H3K9Ac/H3K9me2 ratio in the control cell line are not expressed, but nevertheless are prone for upregulation after the translocation in MCL.

Interesting, in the work (Burman et al., 2015), it has been demonstrated that some histone modifications can predispose genome regions to breakage and translocation by creating more active chromatin state and, hence, more predisposed for double strand breaks (DSB). Thus, we cannot refuse the thought that such distinct active epigenetic signature unraveled in 11q13 locus in normal B cells might increase probability of DSB and therefore - translocations. It is known that most of translocation events in B cells occur during V(D)J IgH gene recombination, a period with high DSB frequency. If that period coincides with time when some other locus has epigenetic background prone for DSB too, it might increase probability of chromosome translocation events by an order of magnitude. In this extend, it is worth to verify whether this active signature in 11q13 genes is specific to B cells, and when exactly during cell cycle it appears.

III - Active chromatin marks are required for abexinostat-triggered effects

Regardless general abexinostat-induced heterochromatin disaggregation, we have not observed global changes in gene expression in response to the treatment. Instead, the changes in histone modifications marks and in gene expression had a precise topography and

affected only small portion of genes. In agreement with our observations, altering a relatively small proportion of expressed genes by HDACis was independently described by other groups (Van Lint et al., 1996), (Peart et al., 2005), (Halsall et al., 2012).

We have discovered that abexinostat effect on histone modifications was largely restricted to loci with active epigenetic background. Namely, the subset of *upregulated* genes or in some cases the entire 11q13 locus, which showed the highest H3K9Ac / H3K9me2 ratio in the genome (**Figure 36** and **Figure 5 in the manuscript**). Perhaps, enrichment in H3K9Ac mark, or in other active mark which coincides with H3K9Ac, might be a requirement for abexinostat-mediated effects in the histones. This is consistent with observations (Lopez-Atalaya et al., 2013) and (Wang et al., 2009), indicating that HDACis effects were largely restricted to loci enriched in active chromatin marks such as H3K4me3, AcH3K9,14 and H4K16Ac.

Why HDACis would be attracted to active chromatin marks? (Wang et al., 2009) suggested a model according to which active and primed genes (not yet expressed, but not silent neither) are subject to dynamic regulation by HATs and HDACs. In actively transcribed regions acetylation marks have to be controlled to avoid its excess, as it can destabilize chromatin and increase cryptic initiation of transcription. In genes primed for transcription, a transient binding of HATs and HDACs was detected indicating their potentiated state for future activation upon receiving external signal. Therefore, as soon as these types of genes are associated with HDACs, they are more sensitive for HDAC inhibitor application. These data is in accordance with our observations, demonstrating that the set of *upregulated* genes, highly expressed in MCL and not expressed but prone for transcriptional activation in normal B cells, reveal intensive response to abexinostat treatment.

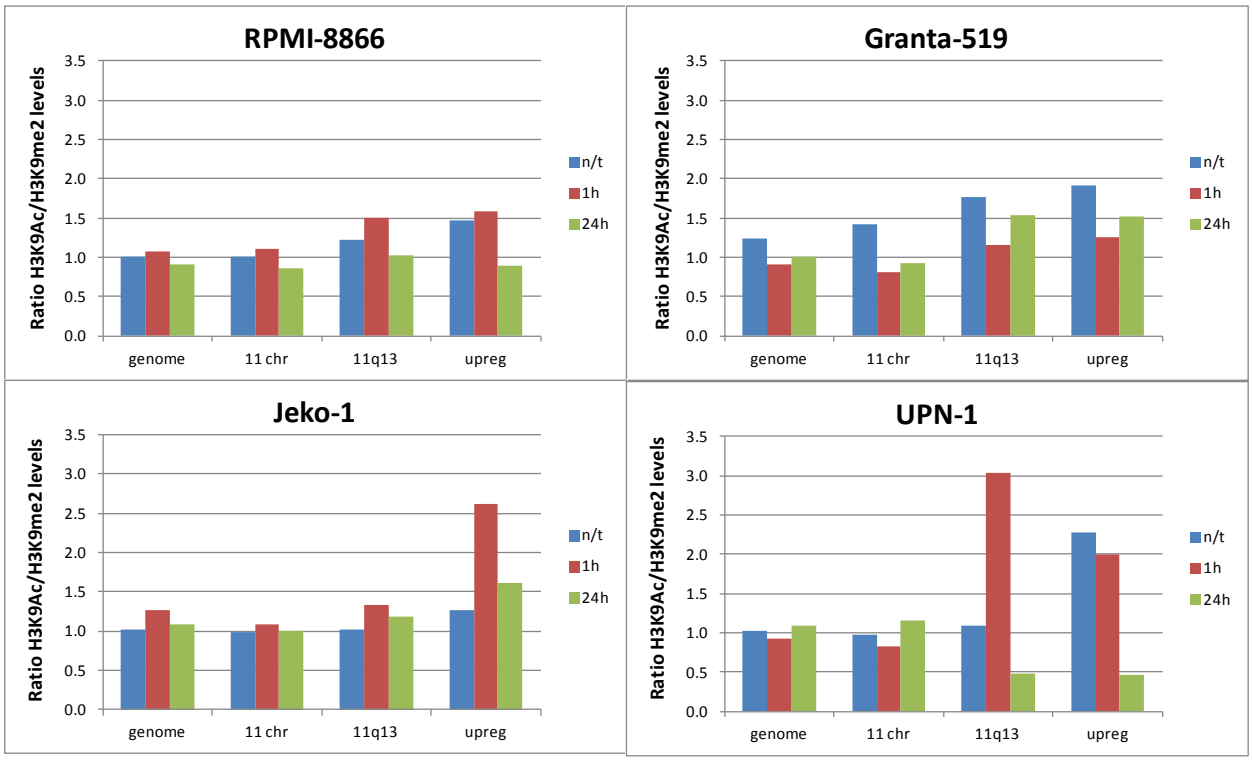


Figure 37. Changes in H3K9Ac/H3K9me2 enrichment in genes' promoters upon abexinostat treatment.

MCL (Granta-519, Jeko-1, UPN-1) and control (RPMI-8866) cells were treated with 100 nM abexinostat and H3K9Ac, H3K9me2 enrichment normalized to panH3 was analyzed at defined time points using Agilent Human Promoter Microarray. Statistically significant H3K9Ac and H3K9me2 peaks were calculated in the entire genome, the 11 chromosome, the 11q13 locus, and in the genes sensitive to upregulation after the translocation t(11;14) ('*upregulated*') for untreated (n/t), and 1h and 24h abexinostat-treated cells. H3K9 acetylation and H3K9 methylation levels were calculated, and then ratio of H3K9Ac/H3K9me2 was taken, which is represented on the charts.

Abexinostat triggered enrichment in H3K9Ac mark *vs.* H3K9me2 in MCL Jeko-1 and UPN-1, and in control RPMI-8866 cell lines. It was detected in short time (1h) in the promoters of *upregulated* genes or in 11q13 locus (**Figure 37**). In contrast, Granta-519 revealed decrease of H3K9Ac / H3K9me2 ratio in 1h. From **Figure 36** is it evident that Granta-519 has especially high enrichment of H3K9Ac *vs.* H3K9me2 mark in entire genome (as compared to other cell

lines), what probably could be a premise of differing HDACi-induced changes in histone modifications. Interesting, shifted H3K9Ac / H3K9me2 enrichment triggered by abexinostat had tendency to be re-balanced in long-time period of treatment (24h), with exception of UPN-1, which demonstrated loss of active mark.

Intriguingly, in MCL, regardless dramatic changes in histone modifications marks in 1h of treatment, we have not detected immediate impact on gene expression. Instead, expression of genes increased only in 24h. Corresponding to a new emergent view on histone code theory, histone-tail covalent modifications do not directly participate in recruitment of chromatin regulators and TFs. Instead, they induce chromatin fiber allosteric transitions which, in turn, play a key role in transcriptional regulation (Lesne et al., 2015), (Rando, 2012). Thus, active chromatin marks would just favor transcriptionally permissive state of a gene, but not trigger transcription. We might suppose that after involving other factors and RNA Pol II, transcription starts with delay after HDACi application. Importantly, in 24h, we have observed overexpression of only a few genes with active epigenetic marks: among the *upregulated* genes, mostly genes with low basal expression reacted to abexinostat with increase in their transcription rates. This does not sound controversial to the pervious reflections: since HATs/HDACs cooperation already provided very high acetylation level to a gene region, HDAC inhibitor apparently cannot increase it even more.

IV - Mechanism of HDACi-induced downregulation of cyclin D1 protein

Data from several research teams have reported HDACi-induced degradation of cyclin D1 protein levels (Heider et al., 2006), (Kawamata et al., 2007), however till now, underlying mechanisms remain obscure.

Consistently with previous observations, our findings showed that incubation of MCL cells with HDACis abexinostat or TSA resulted in downregulation of cyclin D1 protein levels. However, changes in *CCND1* gene expression rates were not observed. It means that HDACi does not downregulate cyclin D1 protein levels via its gene expression. Interesting, we have detected HDACi-induced increase of *CCND1* expression in the control cell lines. But this did not lead to changes in the cyclin D1 protein levels. This is expectedly, as in normal B cells, basal *CCND1* expression was extremely low and protein was expressed in trace amounts, thus HDACi-induced 4-fold increase in transcription did not come to the protein level.

As long as HDACis does not alter *CCND1* expression in MCL, the keys of cyclin D1 downregulation must be lying in the downstream processes. Using 3D-FISH analysis we have analyzed nucleoli state upon abexinostat treatment. Nucleoli are the fabrics of ribosomes where they are synthesized and undergo maturation. Then ribosomal subunits are transported to the cytoplasm to serve for translation of mRNA to proteins. We have found out nucleoli reduction in 24h of abexinostat treatment in both control and MCL cells. However, analysis of 18S+28S rRNA levels did not detect any significant changes during HDACi treatment. Thereby, abexinostat does not induce cyclin D1 protein downregulation on the ribosomal level.

Apart of being ribosomal fabrics, nucleoli have active transcriptional background not only for rDNA genes but as well for RNA Pol II-transcribed genes (Allinne et al., 2014). Thus, from one hand, we can hypothesize that reduction of nucleoli might indicate general depression of transcriptional apparatus in the nucleus. But, from another hand, we detected HDACi-triggered overexpression of some genes in 24h. This apparent contradiction can be explained in appeal to the discussed above gap between expression rates and epigenetic background in a locus. We might speculate that abexinostat probably induces depression of transcriptional background, but this does not yet come to the level of transcription, however it might indirectly lead to redaction of active marks ratio in the histones. Especially this can be true for the genes with high level of active marks, like the discovered subset of *upregulated* genes. Indeed, from the **Figure 37** we can notice that in 24h of treatment ratio of active/not-active histone marks (H3K9Ac/H3K9me2) decreases in 24h as compared to n/t or to 1h in all tested cell lines. This decrease in acetylation levels indicates definitely not direct HDACi effect.

Howbeit, as soon as HDACi-induced downregulation of cyclin D1 protein is not dependent on chromatin modifications, gene expression rates, nor on ribosomal state, we assumed that HDACi might affect cyclin D1 on the level of its degradation. Cyclin D1 turnover is regulated by 26S proteasome (Alao, 2007), thus its inhibition would be expected to increase cyclin D1 protein levels. However, MG132 inhibition of proteasomes, in contrary, led to cyclin D1 levels reduction. In accordance with our results, proteasome inhibitor PS-341-triggered degradation of cyclin D1 protein has been previously described in Mino cell line (Pham et al., 2003). However, in breast cancer cells, proteasome inhibitor PS-341 lead to accumulation of cyclin D1 polyubiquitinated form (Ishii et al., 2006). These observations point to additional non-proteasomal mechanisms of abexinostat action.

In contrast to our findings, HDACi TSA was shown to downregulate cyclin D1 protein levels by 26S proteasomal degradation pathway (Alao et al., 2006). Nevertheless, it was demonstrated on breast cancer cells, which have different molecular-biological background. Moreover, TSA working concentrations were much higher as in our study (1μM *vs.* 6.6 nM).

Recently, (Kawamata et al., 2007) has discovered that in MCL cells, cyclin D1 can be downregulated with HDACi SAHA via blocking protein translation by inhibiting PI3K/eIF4E-BP pathway. Phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR/eIF4E-BP pathway is responsible for initiation of translation (Gingras et al., 2001). Indeed, this pathway was previously shown to be activated in MCL cells and to be linked to elevated levels of cyclin D1 protein (Rizzatti et al., 2005), (Rosenwald et al., 1993). Several investigators have reported that HDAC inhibitors can block the PI3K/Akt pathway and decrease cyclin D1 levels in cancer cells (Zhou et al., 2006), (Kodani et al., 2005), (Zhang et al., 2015), albeit causative role of HDACi in blocking PI3K/eIF4E-BP pathway and hence downregulating cyclin D1 was demonstrated only in the study (Kawamata et al., 2007).

Thus, the same HDACis can decrease cyclin D1 protein levels via different mechanisms depending on the cell type. Our results obtained on MCL indicate mechanism of abexinostat-induced cyclin D1 downregulation rather similar to one described for SAHA in the study (Kawamata et al., 2007). To verify it, analysis of abexinostat effect on PI3K/eIF4E-BP pathway members have to be performed in MCL cells.

MATERIALS AND METHODS

Cell cultures

The human mantle cell lymphoma (MCL) cell lines Granta-519, Jeko-1, UPN-1, Mino, NCEB-1 and follicular lymphoma cell line RL were used in experiments. Either naïve $CD20^+$ B-lymphocytes from peripheral blood or a lymphoblastoid RPMI-8866, Priess, Remb1, IARC-211, IARC-171 cell lines were used as a normal control.

RPMI-8866, Priess, Remb1, IARC-211, IARC-171, Granta-519, Mino, RL cell lines and primary cells from humans were maintained in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 1% penicillin-streptomycin (Invitrogen). UPN-1 and NCEB-1 cells were cultured in MEM alpha medium (Invitrogen, Cergy Pontoise, France) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Perbio science), 2 mM L-glutamine, and 1% penicillin-streptomycin (Invitrogen). Jeko-1 cells were maintained in RPMI 1640 supplemented with 20% fetal bovine serum, 2 mM L-glutamine, and 1% penicillin-streptomycin (Invitrogen). Patients' malignant lymphocytes and normal B-lymphocytes were isolated from peripheral blood, separated by Ficoll-Hypaque density sedimentation, and placed in RPMI-1640 supplemented with 10% serum and antibiotics. Cells were cultured at 37°C in a humidified 5% CO_2 atmosphere.

Drugs treatment

HDAC inhibitors

Stock solution of abexinostat in dimethylsulfoxide (DMSO) at 0.1 mM was conserved at -20°C. Cells were incubated in the appropriate growth media supplemented with abexinostat at 0.1 µM concentration during 1, 24, 48 or 72 hours at normal growth conditions. Control cells had an equivalent level of DMSO added.

Stock solution of TSA 6.6 µM was conserved at -20°C. Working concentration 6.6 nM was used for cell treatment.

Proteasome inhibition

Proteasome inhibitor MG-132 (Euromedex) was prepared as stock solution 25 mM in DMSO and kept at -20°C. Cells were treated in the appropriate growth media supplemented with MG-132 at 25 µM concentration during 24 and 48 hours.

Cell viability assay

Cytotoxic effects of the abexinostat compared to the LBH589 were evaluated on cell proliferation using WST-1 reagent (Roche diagnostic). Different concentrations of the HDAC inhibitor were tested:

0.0002, 0.001, 0.005, 0.0125, 0.025, 0.05, 0.1, 0.2, 0.25, 0.5, 1, 1.25, 1.5 and 2 µM

$5*10^4$ cells were incubated in triplicate with different concentration of abexinostat in tissue culture 96-well-plate. 24h later, 10µl of WST-1 reagent was added to the cells and incubated over 3 hours. Spectrophotometric quantification of cell proliferation was performed at 450 nm. Cell viability was compared to cells incubated with DMSO (<0.02%). Growth inhibition 50 (GI50) was estimated after 24 hours.

Apoptosis assessment

Cell death was assessed by labeling cells with Annexin-V-FITC (Roche Applied Science) and DAPI. Aliquots of 10^6 cells were stained with 0.5µl Annexin-V-FITC for 10 min and diluted in

500µl PBS buffer with 0.5 µl DAPI. Samples were analyzed (n=10 000) by flow cytometry on the FACScan flow cytometer LSRII (BD Biosciences) within 30 min. Annexin V positive cells were considered as apoptotic cells.

Chromatin immunoprecipitation (ChIP)

For chromatin isolation, cells were fixed with 1% formaldehyde and sonicated in a lysis buffer (50mM Tris-HCl, pH 8.0, 10mM EDTA, 1% SDS, 0.2mM PMSF, 1% PIC) with 10 cycles of 20 sec pulse-on, 30 sec pulse-off, 40% amplification. The non-solubilized material was removed by centrifugation at 16,000g for 10 min. The size of chromatin fragments (1-3 nucleosomes) was monitored by electrophoresis in a 1% agarose gel after rev-crosslinking and treatment with 5 µg/ml RNaseA and 2 µg/ml proteinase K.

Chromatin immunoprecipitation was performed as following: 21 µg of chromatin solution was incubated overnight with 25µl of the PrG-dinabeads (Sigma) and 1-5 µg of antibodies in 1ml reaction solution. H3-pan antibodies were used in quantity 1.5 µl per reaction (17-10254, Millipore), H3K9ac 5 µg (17-658, Millipore), H3K9me2 4 µg (ab-1220, abcam) and IgG rabbit as a negative control 2 µg (Millipore). Extracted DNA was washed with ChIP-buffers from ChIP-IT Express kit (Active Motif) and purified by phenol-chlorophorm extraction.

Precipitated DNA was followed by the qPCR and/or microarray analysis.

Immunoprecipitated DNA was qPCR-amplified using hot-start DNA polymerase (FastStart SYBR Green Master Mix, Roche Life Science) and the following primers:

GAPDH Fw : GCT GGC ACC ACT ACT TCA GAG A
Rv : GCC AAC AGC AGA TAG CCT AGG A

GSTP1 Fw : GTG GAG GAA ACT GAG ACC CAC TGA
Rv : TGG AAG GAT GAG AGA CTG CCA CAC
CCND1 Fw : CGC CCT CGG TGT CCT ACT TCA A
Rv : GAT CCC TAG AAA CAC CAC GGC AAA

The presence of non-specific sequences in the ChIP was verified using the following primers:

MYOD Fw : CGC CAG GAT ATG GAG CTA CT
Rv : GAG TGC TCT TCG GGT TTC AG

qRT-PCR

The expression level of 11q13 genes was determined by quantitative real-time PCR using specific primers designed for cDNA listed below. 100 ng of total RNA purified using guanidine thiocyanate and purification columns (NucleoSpin RNA II kit, Machery-Nagel) was converted into cDNA using Random Hexamer Primer (Fermentas) and RevertAid H Minus Reverse Transcriptase (Fermentas). cDNA was quantified using qPCR with FastStart Universal SYBR Green Master (Roche Diagnostics). Expression was calculated with ΔΔCt method (*GAPDH* gene expression used as control).

Gene		**Sequence 5' - 3'**
CD6	Fw	GCC CTG ACC ACC TTC TAC AGT
	Rv	GGG TTG GCA GTT GGG ATG T
CD5	Fw	CCA TCC GTC CTT GAG GTA GA
	Rv	CCT TGT ACC TGC TGG GGA T
MTA2	Fw	TAT GTG GGT GGC TGG TAA TG
	Rv	GCC TGG CTG ATA GTA ATG CC
BAD	Fw	TCA CCA GCA GGA GCA GCC AA
	Rv	GAG CGC GAG CGG CCC CGA AA
KAT5	Fw	CTT GGC CAA AAG ACA CAG GT
	Rv	CAT CCT CCA GGC AAT GAG AT
CTSF	Fw	GAC TGT GAC AAG ATG GAC AA
	Rv	CCA CGG AGT CAT TGA TGT AGA
GSTP1	Fw	AAT GAA GGT CTT GCC TCC CT
	Rv	GAC CTC CGC TGC AAA TAC AT

CCND1	Fw	AGT TGT TGG GGC TCC TCA G
	Rv	AGA CCT TCG TTG CCC TCT GT
UVRAG	Fw	TGG AGT CCC TAG TCC ATG TTG
	Rv	AGG AGG GGA GAA GTT GCA GT
ORAOV1	Fw	GTC AGG ACA TAT TCG ATG CCA T
	Rv	GCT GCC TTC CCT CCA TCA CA
GAPDH	Fw	CTG CAC CAC CAA CTG CTT AG
	Rv	AGG TCC ACC ACT GAC ACG TT

ChIP-on-chip

NimbleGen (tilling micro-array)

After amplification as recommended (Nimblegen), DNA samples were hybridized to a human genome tiling array consisting of 50-mers positioned every 100 bp along non-repeated sequences of the selected regions of chromosomes 11 and 14. Raw data were collected by Nimblegen (Roche-NimbleGen, Iceland). .

The data obtained has been analyzed with the ACME (Algorithm for Capturing Microarray Enrichment) package, available from Bioconductor. ACME analysis and permutation significance testing has been performed as described for the H3K9Me2 and H3K9Ac tiling ChIP data.

Signal intensity data were extracted from the scanned images of each array using Roche NimbleScan software. The ratio of the input signals for the experimental and control samples that were co-hybridized to the array was computed for each feature of the array. Then the log2 ratio was computed. Subsequently, the NimbleGen log2 ratio files have been analyzed using ACME (Algorithm for Capturing Microarray Enrichment) (Scacheri et al., 2006a), (Scacheri et al., 2006b), written in R language and freely available through Bioconductor. ACME supports data analysis for any NimbleGen tiled array design. The software is based on the assumption

that the real signal is represented by multiple probes that are located close to one another in the genome, producing a neighbor effect. After loading the data into R, ACME automatically sorts probes by their genomic location. The user must then set a threshold within the distribution of the ratio measurements above which true positive signals are expected to be enriched. We have set this threshold at 0.85 or the 85th percentile. To identify potential sites of enrichment, a window of user-defined size (window is of size 400, corresponding to 1-2 nucleosomes) moves stepwise along the tiled region, centering at every probe and testing if it contains a higher than expected number of probes over the defined threshold 0.85 (chi-squared test). Single probes that yield high intensity ratios most likely represent noise and are automatically filtered out by the windowing/threshold analysis (Scacheri et al., 2006b). The resulting output contains treated p-values ($-\log_{10}$(p-value)) with corresponding chromosome coordinates. We have imported these results into the Integrated Genome Browser (Nicol et al., 2009) for visualization. Analyses performed at other window sizes and threshold produced similar results (data not shown).

While computing p-values using ACME, independence between individual data points was violated to an unknown degree (Scacheri et al., 2006a), (Scacheri et al., 2006b). We used a permutation-based algorithm to assess the significance of the ACME p-values and to establish relevant cutoffs likely to be representative for H3K9Me2 and H3K9Ac binding events. The null hypothesis is that the probe label order has no effect upon the computed ACME p-values. If changing the order of the probe labels destroys the effect, then a random permutation test can be done. Hence, we have generated 40 permuted samples for each of the two datasets (H3K9Me2 and H3K9Ac). We processed the ChIP-on-chip signal with ACME for each of the permuted datasets, and then computed the False Discovery Rates (FDR), corresponding to the ACME p-value of each probe. FDR was computed by counting the rate of permutation ACME

p-values which were larger than the ACME p-value obtained on the real dataset. According to the obtained results, for p-value <0.0001 ($-\log_{10}$(p-value)>4), the FDRs were very close to zero, meaning that peaks characterized by $-\log_{10}$(p-value)>4 were not likely to be discovered by chance. Thus, we set cutoff p-value = 0.0001.

Agilent micro-array (gene promoter zones)

After amplification, DNA samples were hybridized to the two-colored SurePrint G3 Human Promoter Microarray, 1x1M (G4873A, Agilent, Palo Alto, Calif., USA) covering genes' promoter zones all over the genome. Labeling, hybridization and washing were carried out according to the Agilent mammalian ChIP-chip protocol (ver.9.0).

Scanned images were quantified with Agilent Feature Extraction software under standard conditions. The probe signals were filtered: replicated probes were merged by median and saturated probes (at least 1 channel) with high pixel heterogeneity have been removed. Filtered probes were processed as following: intra-array quantile normalization, log2 ratio transformation (ratio of modified histone probe signal to pan-H3 histone probe signal), GC% normalization, Z-score transformation to homogenize the value distributions (median was subtracted from each log2(ratio) and result was divided by the standard deviation).

The p-value for each probe was computed by a modification of the Whitehead algorhythm as follows: an average was calculated for each probe with 2 surrounding it probes within 300bp, the distribution of these averages was obtained, each average value was reported to the distribution of averages. The area to the right of the value under the averages distribution curve was computed: this is the p-value which was attached to each probe. The threshold is set at

95% of the distribution (p-value = 0.05). The resulting output contains treated p-values (-log10(p-value)) with corresponding chromosome coordinates. We have imported these results into the Integrated Genome Browser (Nicol et al., 2009) for visualization.

3D-fluorescence in situ hybridization and immunodetection

Cells were immobilized on glass coverslips coated with Poly-D-lysine hydrobromide (Sigma). The cells were then treated as previously described to preserve their three-dimensional (3D) structure (Solovei et al., 2002). Denatured nuclei were hybridized overnight with denatured probes, *IgH* labeled green (RP11-346I20, AmpliTech) and *CCND1* labeled orange (RP11-300I6, AmpliTech). After probe hybridization, slides were washed and nucleoli were detected using mouse anti-B23 antibody (Sigma) and goat anti-mouse Pacific Orange antibody (Invitrogen). Transcription factories or heterochromatin clusters were immunodetected simultaneously with detection of nucleoli using rabbit anti-RNA Pol II phosphorylated at serine 5 (ActiveMotif) or rabbit anti-H3K9me3 (Upstate) antibodies, correspondingly, and goat anti-rabbit Alexa 633 (Invitrogen) antibody. DNA was counterstained with 4,6 diamidino-2-phenylindole (Vectashield, Vector) or Bobo1 (Invitrogen). Confocal microscopy, image processing, and statistical analysis were carried out as described (Allinne et al., 2014).

Flow cytometry analysis

For Cyclin D1 protein level estimation 10^6 cells were fixed and permeabilized with pure cold methanol over night. Fixed cells were incubated with cyclin D1 FITC-conjugated mouse monoclonal antibody (Santa Cruz sc-8396) as recommended by the manufacturer. Cellular

fluorescence was measured on the FACScan flow cytometer LSRII (BD Biosciences) with the use of corresponding software.

B-cell activation

For the control B-cell activation experiments, B-cells (105 cells/ml of complete medium) were activated by incubation for 24h, 48h and 72h with a cocktail containing 10 ng/ml recombinant human IL4 (Sigma), 1 µg/ml anti-human monoclonal antibodies to CD40 (G28.5 clone, Biolegend), and 20 µg/ml monoclonal anti-human IgM (clone DA4.4, kindly provided by Joelle Wiels). Activation was confirmed in 24h of treatment by FACS with PE-labeled anti-CD23, PerCP/Cy5.5-labeled anti-CD69, and APC-labeled anti-CD80 B-cell activation markers, and FITC-labeled anti-CD19 B-cell specific marker (Biolegend). All the FACS antibodies were produced by Biolegend (USA). B-cell activation was further confirmed by BrDU incorporation, 72h after the activating treatment. For BrDU incorporation cells were incubated with 50 µM of BrdU for 1 hour at 37°C. After that cells were washed with PBS and fixed in cold 70 % ethanol using standard procedure. Cell cycle was analyzed by FACS with propidium iodide for DNA staining.

Analysis of gene expression data from GEO database

MCL genes expression data collected from Gene Expression Omnibus (GEO) and corresponding to different microarray platforms, was compared to gene expression in normal naïve B (NNB) cells. The raw expression data from GEO was downloaded: MCL (15 conventional MCL samples composed of Peripheral blood cd19+ tumour cells, GSE16455) and

NNB (3 naïve B cell samples from tonsils of patients undergoing routine tonsilectomy, GSE12366). Student *t*-tests on the three probes targeting *GAPDH* showed that there was no difference in the expression levels of *GAPDH* in the MCL group versus the NNB group. Barplots showed same ranges among the two groups too, hence no batch effect correction has been carried out.

The expression data have been normalized (R function rma) and half of the probes corresponding to the least variable features have been removed (R function nsFilter, default settings). The differentially expressed genes between MCL and NNB lymphocytes were identified using an Empirical Bayesian approach to computing a moderated t-statistic variable implemented in the limma R package with the Benjamini-Hochberg procedure for multiple test adjustment. The adjusted p-value threshold was set to 0.05. Next, we have used Bioconductor library GexMap (Cagnard, 2009) in order to detect the existence of chromosomic regions, characterized by clusters of differentially expressed genes. With this library, the list of differentially expressed genes was mapped to the respective chromosomal locations, chromosome by chromosome. Each chromosome is divided into units 1 Mbp and the quantity of observed differentially expressed genes is compared to a computed theoretical (expected) quantity of differentially expressed genes using the following equation:

$$Expected_{diff\_\exp/unit} = ENSEMBL_{genes/unit} \frac{\sum_{all\_units} Observed_{diff\_\exp/unit}}{\sum_{only\_units\_with\_diff\_\exp\_genes} ENSEMBL_{genes/unit}}$$

ANNEX

Perinucleolar relocalization and nucleolin as crucial events in the transcriptional activation of key genes in mantle cell lymphoma.

Allinne J, Pichugin A, Iarovaia O, Klibi M, Barat A, Zlotek-Zlotkiewicz E, Markozashvili D, Petrova N, Camara-Clayette V, Ioudinkova E, Wiels J, Razin SV, Ribrag V, Lipinski M, Vassetzky YS.
Blood. 2014 Mar 27;123(13):2044-53. PMID: 24452204

In this paper we were analyzing the molecular mechanism of *CCND1* activation caused by t(11;14) translocation in mantle cell lymphoma (MCL).

Using a combination of 3D- and immuno-FISH, we have first observed that translocated *CCND1* allele significantly more distant from the nuclear membrane than its non translocated counterpart, with a very high proportion of *IgH-CCND1* chromosomal segments localized next to a nucleolus. Regardless of their subnuclear localization, gene loci can be protected from nearby activating elements by insulator sequences such as those bound by the CCCTC-binding factor. Using the Transfac software, 2 potential CTCF-binding sites, referred to here as CTCF26 and CTCF2, were identified 26 and 2 kb upstream of the *CCND1* promoter.

My role in the paper consisted in performing chromatin immunoprecipitation experiments. The precipitated DNA fragments were analyzed by quantitative PCR using primers for amplification of several sequences located near or within of *CCND1* gene. This analysis confirmed the presence of CTCF on 2 potential CTCF-binding sites - CTCF26 and CTCF2 (Figure 5 in the article).

However, despite CTCF being bound immediately upstream of *CCND1* transcription start site, that gene is still transcribed in MCL cells. With further analysis we have shown that nucleolin, a nucleolus-derived factor, interacts with potential enhancer elements located further

downstream in the *CCND1* gene and activates transcription. That coincided as well with colocalisation of active RNA polymerase II close to perinucleolar areas.

I have also tested whether the proximity with a nucleolus is important for *CCND1* transcription in MCL cells using a pan-histone deacetylase inhibitor Abexinostat (S78454 / PCI-24781). It provokes an overall disorganization of the nuclear structure. In treated GRANTA-519 MCL cells the percentage of the der14-carried *CCND1* alleles, still localized next to a nucleolus, decreased from 75% to 53% as early as 1 hour after treatment. Next, I have performed gene expression analysis using RT-qPCR and shown that this phenomenon was accompanied by a 4-fold reduction in *CCND1* transcription (Figure 6 in the article). Thus, *CCND1* expression in MCL cells requires an unperturbed chromatin and nuclear organization.

Our results allowed us to propose a hypothesis that *CCND1* transcriptional activation in MCL cells relates to the repositioning of the rearranged *IgH-CCND1*-carrying chromosomal segment in a nuclear territory with abundant nucleolin and active Pol II molecules. Similar transforming events could occur in Burkitt's and other B-cell lymphomas.

Regular Article

LYMPHOID NEOPLASIA

Perinucleolar relocalization and nucleolin as crucial events in the transcriptional activation of key genes in mantle cell lymphoma

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Key Points

- We propose a novel oncogenic mechanism linked to the perinucleolar relocalization of chromosomal segments resulting from the translocation.
- MCL and BL translocations result in new *Ccnd1* and *c-myc* nuclear positioning, respectively, and nucleolin-dependent activation in both cases.

In mantle cell lymphoma (MCL), one allele of the cyclin D1 (*Ccnd1*) gene is translocated from its normal localization on chromosome 11 to chromosome 14. This is considered as the crucial event in the transformation process of a normal naive B-cell; however, the actual molecular mechanism leading to *Ccnd1* activation remains to be deciphered. Using a combination of three-dimensional and immuno-fluorescence in situ hybridization experiments, the radial position of the 2 *Ccnd1* alleles was investigated in MCL-derived cell lines and malignant cells from affected patients. The translocated *Ccnd1* allele was observed significantly more distant from the nuclear membrane than its nontranslocated counterpart, with a very high proportion of *IgH-Ccnd1* chromosomal segments localized next to a nucleolus. These perinucleolar areas were found to contain active RNA polymerase II (PolII) clusters. Nucleoli are rich in nucleolin, a potent transcription factor that we found to bind sites within the *Ccnd1* gene specifically in MCL cells and to activate *Ccnd1* transcription. We propose that the *Ccnd1* transcriptional activation in MCL cells relates to the repositioning of the rearranged *IgH-Ccnd1*–carrying chromosomal segment in a nuclear territory with abundant nucleolin and active PolII molecules. Similar transforming events could occur in Burkitt and other B-cell lymphomas. (*Blood*. 2014;123(13):2044-2053)

Introduction

Many non-Hodgkin B-cell tumors are strongly associated with recurrent reciprocal chromosomal translocations. These include follicular lymphoma (FL), Burkitt's lymphoma (BL), and mantle cell lymphoma (MCL), 3 malignancies in which the rearrangement most often implicates the immunoglobulin heavy chain (*IgH*) locus on chromosome 14 along with the *Bcl2* antiapoptotic gene, the *c-myc* oncogene, or the cyclin D1 (*Ccnd1*) gene, respectively. Contrasting with the basal expression level of *c-myc*, both *Bcl2* and *Ccnd1* are silent in normal B cells. The molecular mechanisms at work in these various B-cell proliferations vary in relation with the stage of maturation of the concerned B lymphocyte as well as with the location of the cell when the initial cell transformation event occurs.[1,2] Whereas BL and FL arise from germinal center B cells at various stages of maturation, MCL is believed to result from accidental *IgH* recombination events affecting B lymphocytes at the pre-B stage of differentiation in the bone marrow.[3] Following the prototypical t(11;14)(q13;q32) rearrangement observed in the vast majority of MCL, one *Ccnd1* gene allele becomes juxtaposed with the *IgH* locus on the derivative chromosome 14 (der14) and *Ccnd1* gets constitutively expressed.[4] It is generally assumed that this relies on the action of the potent Eμ enhancer present upstream of *Ccnd1* in the *IgH* locus. The distance between the *IgH* and *Ccnd1* genes can reach several hundreds of kilobases,[5,6] however, and the question remains as to the actual molecular basis for *Ccnd1* activation in MCL cells.

In a cell nucleus, intact chromosomes occupy specific territories.[7,8] As a rule, but with a few exceptions,[9] gene-dense chromosomes and actively transcribed chromosomal segments packaged in euchromatin tend to be more centrally located than gene-scarce, transcriptionally inactive heterochromatin segments.[10-12] Chromosomal rearrangements such as those observed in hematopoietic malignancies necessarily trigger changes in the nuclear positioning of numerous genes.

Here, we have investigated the consequences of the chromosomal translocation on the position of the 2 alleles of the *IgH* and *Ccnd1* genes in the nucleus of MCL cells. The *IgH* and *Ccnd1* genes juxtaposed on the der14 were found more centrally located than the corresponding alleles on the nonrearranged chromosomes, and

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J.A., A.P., and O.I. contributed equally to this study.

The online version of this article contains a data supplement.

There is an Inside *Blood* commentary on this article in this issue.

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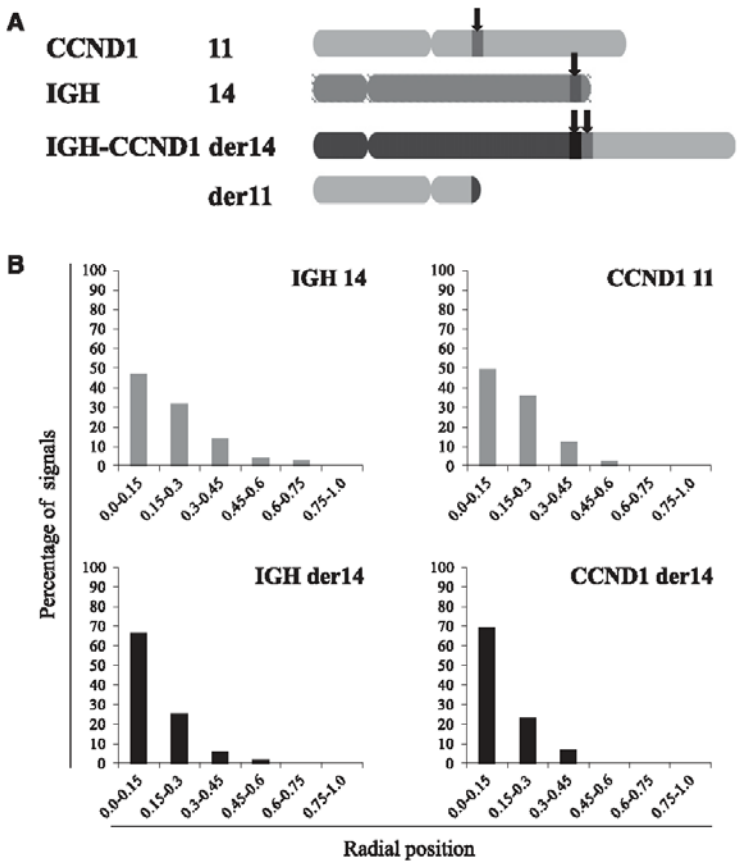

Figure 1. Repositioning of *IgH* and *Ccnd1* in MCL cells. FISH was performed on 3-dimensionally preserved Granta-519 cell nuclei to reveal the *IgH* and *Ccnd1* alleles. (A) Schematic representation of the chromosomes involved in the t(11;14) translocation in MCL cells and localization of the *IgH* and *Ccnd1* probes used. (B) Distribution of the translocated (der14) and nontranslocated *IgH* and *Ccnd1* alleles according to their radial positioning in Granta-519 cell nuclei. Distances were measured as described in Materials and methods. Histograms represent the percentage of FISH signals in the nuclear space with the range 0.0 to 0.15 corresponding to the centermost fraction of the nucleus and 0.75 to 1 to its periphery. Each graph represents a minimum of 120 FISH signals.

they were located very close to at least one nucleolus. Active RNA polymerase II (PolII) molecules were detected in these perinucleolar areas. We propose that the transcriptional activation of *Ccnd1* in MCL cells directly relates to the nuclear repositioning of one *Ccnd1* allele close to a nucleolus via *Ccnd1* interaction with a nucleolus-abundant transcription factor nucleolin. Such a mechanism could also take place in other B-cell proliferations.

Materials and methods

Cells

The MCL-derived cell lines Granta-519, Jeko, UPN1, and UPN2 and the Raji and P3HR1 BL cell lines were grown as described in supplemental Methods. Patients' and control lymphocytes were isolated from peripheral blood, separated by Ficoll-Hypaque density sedimentation, and placed in RPMI-1640 supplemented with 10% serum and antibiotics. HeLa and HeLaS3 cells were grown as described.[13] Transient transfection of HeLa cells was performed using Lipofectamine2000 according to the manufacturer's instructions.

Ethics statement

The human samples were obtained with the written consent of patients in accordance with the national legislation. This study was conducted in accordance with the Declaration of Helsinki.

3D-fluorescence in situ hybridization and immunodetection

Cells were immobilized on glass coverslips coated with Poly-D-lysine hydrobromide (Sigma). The cells were then treated as previously described to preserve their three-dimensional (3D) structure.[14] Denatured nuclei were hybridized overnight with denatured probes, either IGH labeled green (RP11-346I20, AmpliTech) and CCND1 labeled orange (RP11-300I6, AmpliTech) for t(11;14) or IGH labeled green and MYC labeled red (RP11-80K22, AmpliTech) for t(8;14). After probe hybridization, slides were washed and nucleoli were detected using mouse anti-B23 antibody (Sigma) and goat anti-mouse Pacific Orange antibody (Invitrogen). Transcription factories or heterochromatin clusters were immunodetected simultaneously with detection of nucleoli using rabbit anti-RNA PolII phosphorylated at serine 5 (ActiveMotif) or rabbit anti-Histon H3 tri-methylated at lysine 9 (Upstate) antibodies, correspondingly, and goat anti-rabbit Alexa 633 (Invitrogen) antibody. DNA was counterstained with 4,6 diamidino-2-phenylindole (Vectashield, Vector) or Bobo1 (Invitrogen). Confocal microscopy, image processing, and statistical analysis were carried out as described elsewhere[15-17] and in supplemental Methods.

Nuclear extracts and EMSA

Nuclear extracts were prepared from HeLaS3 cells, and electrophoretic mobility shift assay (EMSA) assay was carried out as described in supplemental Methods.

Plasmids

The pEGFPC1Nucleolin nucleolin expression plasmid expressing nucleolin[18] was a kind gift of Dr J. Borowiec. The plasmids used for the luciferase

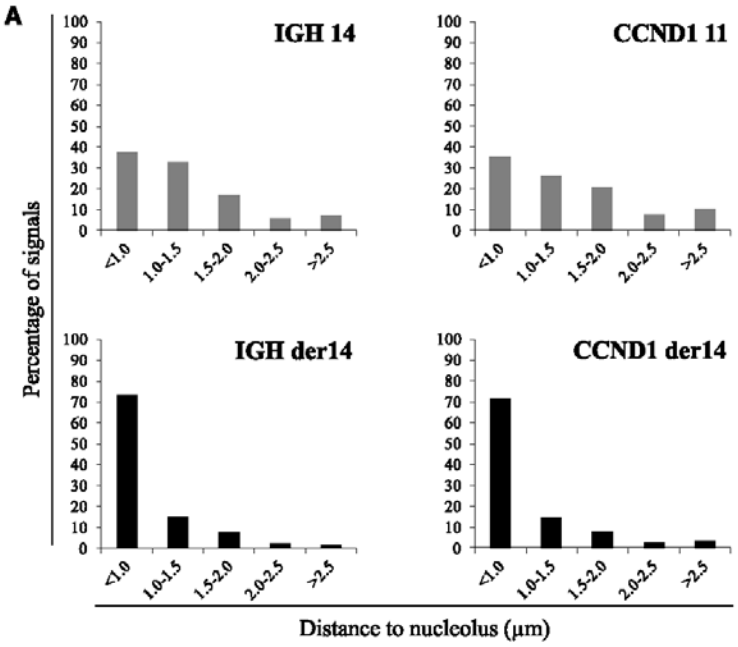

Figure 2. Perinucleolar relocalization of the der14 *IgH* and *Ccnd1* alleles in the MCL Granta-519 cell line. The *IgH* and *Ccnd1* alleles and nucleoli were revealed by immuno-FISH. (A) Distances to the closest nucleolus measured as described in Materials and methods for a minimum of 120 FISH signals. (B) A representative image of double-labeled DNA FISH in Granta-519 cell line. IgH is green, Ccnd1 is red, and nucleolus (B23) is blue. Scale bar represents 5 μm.

activity were derived from the 1748D1Luc plasmid (a gift of Dr B. Sola), renamed pD1Luc. The putative LR1 sites were synthesized in vitro and cloned into the *Bam*H1 restriction site of the 1748D1Luc: LR1.1 5′-GATCCTGG CCTCTCCCAGGCTGGGCCACCTGCCAGG-3′; LR1.2 5′-GATCCAT CTCTCCCCAGCCTTGACCCCAATAAGG-3′; and LR1.3 5′-GATCC GAGCCATGGGTCCAGCCTGACCCGCATG-3′.

Reporter gene assays

Luciferase activity was determined 48 hours after transfection with luciferase reporter plasmid using the Dual Luciferase Assay (Promega) and normalized to protein concentration and to the activity of the phRL-TK reporter (Promega). All transfections were performed in triplicates and repeated in 3 independent experiments. Figures show the average result of 3 independent experiments.

Reverse transcription and quantitative polymerase chain reaction (PCR)

Total RNA was isolated from 2 × 10^6 HeLa cells transfected with the pEGFPC1Nucleolin expression plasmid[18] and from mock-transfected HeLa cells using Trizol (Invitrogen) and reverse transcribed using the High Capacity cDNA Archive kit (Applied Biosystems). cDNA was amplified using specific primers and the Taqman PCR mix (Applied Biosystems). Expression was analyzed using the ΔΔCt method. The primers used for amplification are listed in supplemental Methods.

ChIP

Chromatin immunoprecipitation (ChIP) was performed starting from Granta-519 cells and normal human lymphocytes using the ChIP-IT Express kit (Active Motif) and antibodies against CTCF (Abcam) nucleolin (Sigma) and acetylated and methylated forms of H3 (Abcam) according to the manufacturer's protocol. Immunoprecipitated DNA was PCR-amplified using specific primers listed in supplemental Methods.

ChIP-on-chip

DNA samples were hybridized to a human genome tiling array consisting of 50-mers positioned along nonrepeated sequences of the selected regions of chromosomes 8, 11, 14, 16, and X. Raw data were collected by Nimblegen (Roche-NimbleGen). The results were deposited in the GEO database. ChIP-on-chip analysis is described in supplemental Methods.

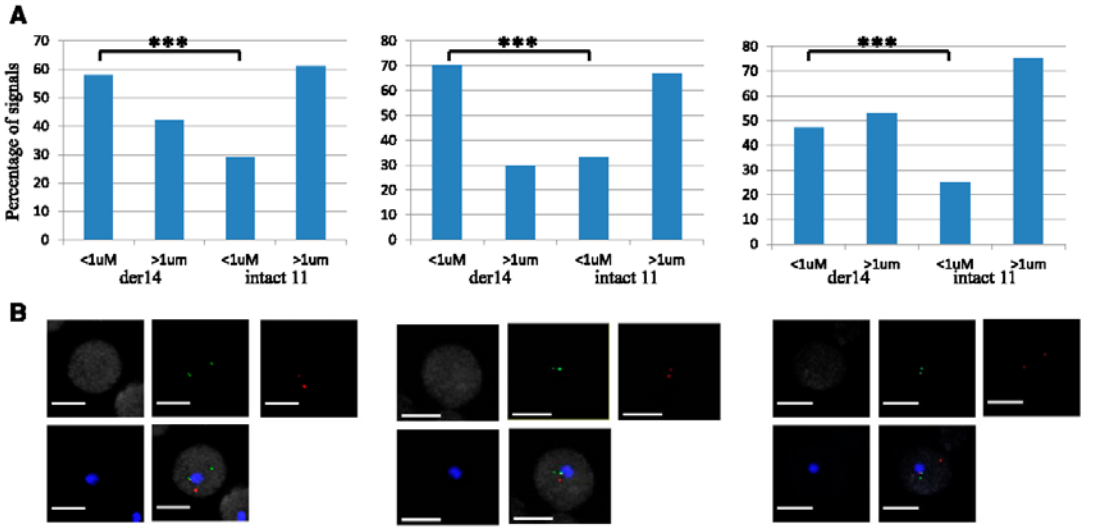

Figure 3. Perinucleolar relocalization of the translocated Ccnd1 allele in MCL patients. (A) Graphs show the distance of FISH signals to the nucleoli. Each graph corresponds to one patient with MCL. Distances have been measured in at least 100 nuclei. ***$P < .001$. (B) Representative images of double-labeled DNA FISH for each patient. IgH is green, Ccnd1 is red, and nucleolus (B23) is blue. Immuno-FISH and distance measurements were performed as in Figure 2, specifically on cells with the t(11;14) translocation, which were detected by colocalization of the IgH and Ccnd1 signals in the nuclei. Scale bar represents 5 μm.

Results

The translocated *Ccnd1* allele relocalizes close to a nucleolus

In MCL cells, the balanced t(11;14)(q14q32) translocation moves one *Ccnd1* allele from its normal position on chromosome 11q to a position on the der14. We wondered whether this translocation was associated with a change in the nuclear positioning of the *Ccnd1* and *IgH* genes. To analyze their radial positioning, cells from the MCL-derived Granta-519 cell line were analyzed by 3D-fluorescence in situ hybridization (FISH) using specific probes (Figure 1A). For each signal, the minimal distance to the nuclear membrane was measured in the 3-dimensional space of the nucleus. From the juxtaposed signals they produced, the *IgH* and *Ccnd1* loci on the der14 were readily and systematically identified in every cell. Results reported in Figure 1B indicate that for each gene, the distribution of each of the 2 alleles differed according to whether *Ccnd1* and *IgH* lay on a nonrearranged chromosome or on the der14. Thus, for both genes, the percentage of alleles in the centermost radial position was significantly higher when on the der14 than on the intact 14 (*IgH*, 67% vs 46%) or 11 (*Ccnd1*, 70% vs 49%) ($P < .001$). Similar results were obtained in UPN1 and Jeko, 2 other MCL cell lines tested, as well as in fresh lymphoma cells from MCL patients (supplemental Figure 1) as shown in Figure 3. Having observed this relocalization, we next asked whether the juxtaposed *IgH* and *Ccnd1* alleles had been displaced toward a specific subnuclear domain. Indeed, the der14 *IgH* and *Ccnd1* signals appeared to concentrate in the periphery of nucleoli, as previously noticed.[19] To quantify this observation, distances to the closest nucleolus were measured for both alleles of the *IgH* and *Ccnd1* genes. The distributions differed strikingly between normal and rearranged alleles (Figure 2; supplemental Figure 1). Thus, the der14 *IgH* and *Ccnd1* signals were located <1 μm away from a nucleolus in >72% of the nuclei vs only ~37% for their counterparts on intact chromosomes 11 and 14 (Figure 2; $P < .001$). Likewise, in lymphoma cells from 3 patients with MCL (Figure 3) as well as in the UPN1 and Jeko cell lines (supplemental Figure 1), the der14 *Ccnd1* gene allele was found to lie close to the nucleolus much more frequently (58%, 70%, and 47% of the signals in each of the 3 patients) than the second allele on the nonrearranged chromosome (29%, 33%, and 24%, respectively; $P < .001$).

The rearranged *IgH-Ccnd1*–carrying chromosomal fragment associates with perinucleolar transcription factories, not heterochromatin

Ccnd1, which is not expressed in normal naive B cells, has been found expressed in a monoallelic fashion in cancer cells.[19,20] Because the *IgH-Ccnd1* chromosomal fragment on der14 chromosome is located close to a nucleolus, we wondered whether *Ccnd1* transcription would actually take place in perinucleolar territories that are not known for being prone to PolII-dependent gene transcription. To test this, Granta-519 cells were stained using the B23 antibody that decorates nucleoli and an antibody to the phosphorylated C-terminal domain of the transcriptionally active form of PolII. Clusters of active PolII molecules were indeed readily detected in the immediate vicinity of nucleoli (Figure 4A). This was compatible with gene transcription taking place in this particular nuclear territory. In each cell, the proximity between the *IgH-Ccnd1* chromosomal fragment and activated PolII foci was then evaluated. Signals from *IgH* (Figure 4Bi, green), *Ccnd1* (Figure 4Bii, red), and PolII (Figure 4Biii, white) often coincided in a location immediately adjacent to a nucleolus (Figure 4Biv, blue). This can be clearly seen in the overlays superimposing the *IgH*, *Ccnd1*, and nucleolus with (Figure 4Bvi) or without (Figure 4Bv) the PolII staining. In contrast, the *Ccnd1* signals generated from the intact chromosome 11 almost never colocalized with a PolII transcription factory (data not shown), consistent with the monoallelic expression of *Ccnd1*.

We then simultaneously visualized the *IgH* and *Ccnd1* genes, nucleoli, and heterochromatin (H3K9me3). One example of such an

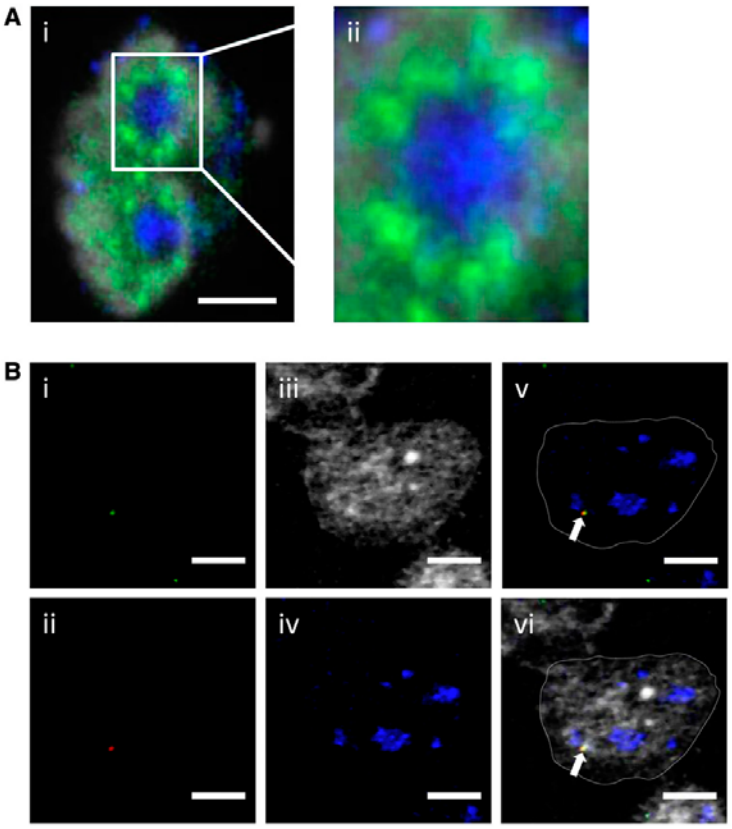

Figure 4. Simultaneous detection of fluorescently labeled transcription factories, nucleoli, heterochromatin, *IgH*, and *Ccnd1* alleles in the Granta-519 MCL cell line. (A) (i) Active RNA PolII (green) and nucleoli (B23, blue) were immunostained with the nucleus counterstained with 4,6 diamidino-2-phenylindole (gray); (ii) enlargement focused on the nucleolus. Scale bar represents 3 μm. (B) *IgH* (green, i), *Ccnd1* (red, ii), active RNA PolII (gray, iii), and nucleoli (B23, blue, iv) were simultaneously revealed by immuno-FISH. In merged images shown in (v) (merged from i, ii, and iv) and (vi) (merged from i, ii, iii, and iv), the arrow points to the der14-carried *IgH* and *Ccnd1* alleles. Scale bar represents 3 μm.

analysis is displayed in supplemental Figure 3 with the 4 profiles generated along an axis shown in supplemental Figure 3i. As expected, the *IgH* (green) and *Ccnd1* (red) peaks were almost superimposed, consistent with their juxtaposition on the der14. The nucleolus fluorescence (blue) lay very close to the *IgH-Ccnd1* double peak. The heterochromatin fluorescence profile (gray) appeared as alternating peaks and ditches, with one ditch precisely corresponding to the *IgH-Ccnd1* double peak. Taken together, these results confirmed the juxta-nucleolar localization of the *IgH-Ccnd1* chromosomal fragment and its association with PolII transcription factories interspersed between heterochromatin-rich areas.

CTCF binds upstream of the *Ccnd1* gene in MCL cells

Regardless of their subnuclear localization, gene loci can be protected from nearby activating elements by insulator sequences such as those bound by the CCCTC-binding factor CTCF (for review see Wallace and Felsenfeld[21]). Using the Transfac software,[22] 2 potential CTCF-binding sites, referred to here as CTCF26 and CTCF2, were identified 26 and 2 kb upstream of the *Ccnd1* promoter (Figure 5A). Their potential as CTCF-binding sites was tested in EMSA experiments. A strong retardation signal was produced in the presence of CTCF2 (Figure 5B lanes 2-3). The electrophoretic retardation of labeled CTCF2 was abolished in the presence of a 10× excess of FII, the canonical CTCF site (lanes 4-5), or with cold CTCF2 (lanes 8-9) but not CTCF26 (lanes 6-7). The presence of CTCF was confirmed by ChIP experiments on Granta-519 cell line and normal lymphocytes. In control lymphocytes, the CTCF-specific antibody precipitated the chromatin fragment containing CTCF2, but not those containing CTCF26 or the negative control sequences (Figure 5C). This sharply contrasted with the ChIP results in Granta-519 cells, where a 21-fold enrichment was obtained with the CTCF2-containing chromatin fragment compared with <5-fold in normal lymphocytes. Thus, the CTCF-binding element located 2 kb upstream of the transcription start site of *Ccnd1* is indeed occupied by CTCF in MCL cells and to a lesser extent in normal lymphocytes. This may play a role both in insulation of the *Ccnd1* promoter and in transcriptional regulation via CTCF.

Nucleolin binds the *Ccnd1* gene and activates its transcription

Because in MCL cells, the *Ccnd1* gene is transcribed despite CTCF being bound immediately upstream of its transcription start site, we reasoned that a nucleolus-derived factor interacting with potential enhancer elements located further downstream in the *Ccnd1* gene would be well suited to play a role in transcriptional activation. One of the most abundant components in nucleoli is nucleolin, a multifunctional protein implicated in transcription regulation and other nuclear processes. Together with HnRNP-D, nucleolin forms a site-specific DNA-binding heterodimer known as LR1, whose transactivating activity is crucial for normal B-cell development.[23] During B-cell differentiation, intragenic recombination and class switch require the binding of LR1 to specific nucleotide sequences in the switch region of the *IgH* locus.[24] We have detected 3 putative LR1-binding

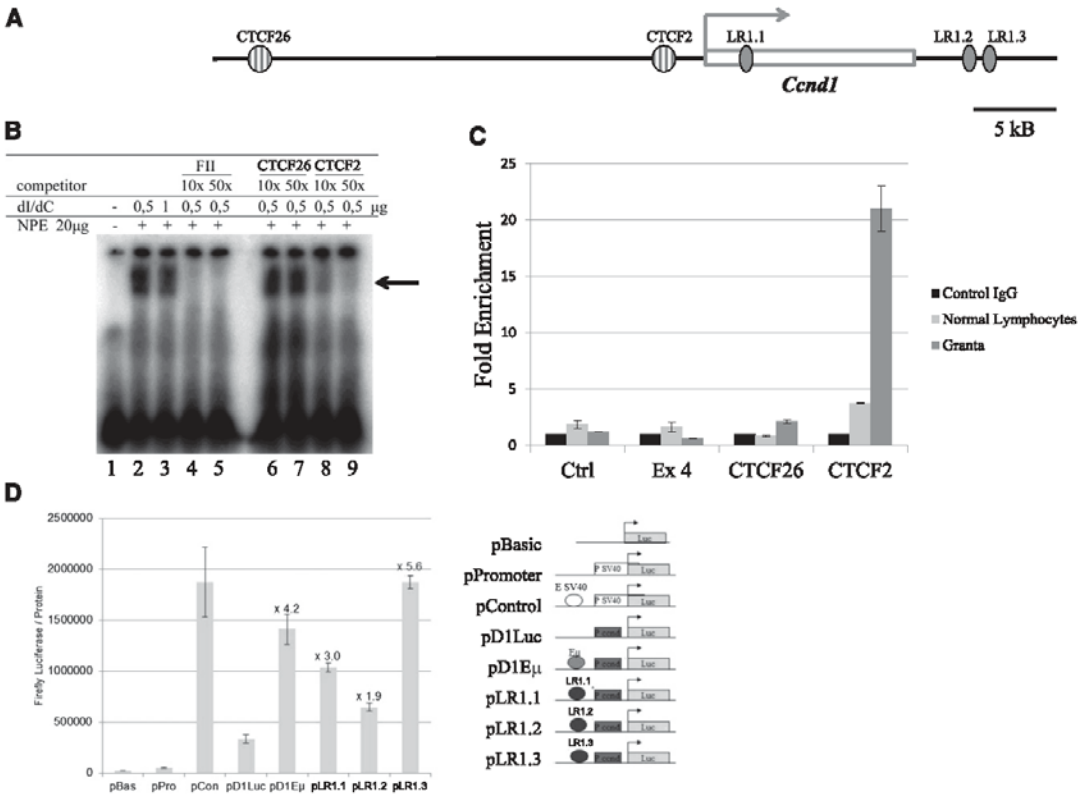

Figure 5. CTCF- and LR1-binding sites in the *Ccnd1* gene. (A) Schematic representation of putative CTCF- and LR1-binding sites within and around the *Ccnd1* gene. (B) Gel retardation assay using a labeled oligonucleotide corresponding to the CTCF2 sequence. dI/dC, unlabeled nonspecific competitor; FII, canonical CTCF site[34]; NPE, nuclear protein extract prepared from HeLa cell nuclei. CTCF2 and CTCF26 indicate unlabeled oligonucleotides tested for their capacity to compete for labeled CTCF2. The arrow points to the position of the CTCF2-specific band. (C) CTCF binding in vivo. ChIP was performed on Granta-519 cells and normal lymphocytes using anti-CTCF dark and light gray bars, CTCF (Ab) or a nonspecific antibody (black bars, ctrl IgG). The precipitated DNA fragments were analyzed by quantitative PCR using primers for amplification of sequences located ~70 kb centromeric to the *Ccnd1* gene or within its exon 4, neither being recognized by CTCF (immunoprecipitation negative controls, "ctrl" and "Ex4"); for CTCF26 and CTCF2, the 2 potential CTCF binding sites identified in *Ccnd1*. Error bars represent the standard deviation between duplicates within the same experiment. (D) LR1-binding sites in the *Ccnd1* gene as transcriptional enhancers. The transcriptional activity of the 3 potential LR1-binding sites identified in the *Ccnd1* gene was tested in HeLa cells 48 hours after transfection. The enhancer strength was quantified relative to the luciferase activity generated with the reference SV40 promoter.

sites within or in the vicinity of the *Ccnd1* gene, one intronic in the body of the gene (LR1.1), the other 2 (LR1.2 and LR1.3) immediately downstream of the *Ccnd1* coding sequence (Figure 5A). Each site was cloned into a luciferase-expressing reporter and tested in HeLa cells for its capacity to enhance basal transcription. Luciferase expression increased 1.9- to 5.6-fold in cells transfected with each of the 3 LR1 site-containing constructs, comparable with the 4.2-fold increase obtained using the positive control Eµ enhancer from the *IgH* gene (Figure 5D). Thus, the nucleolin/LR1 binding sequences present in the *Ccnd1* gene behave as bona fide transcriptional enhancers, consistent with the an activating role in *Ccnd1* transcription in MCL cells.

To further investigate this possibility, ChIP-on-chip experiments were performed on Granta-519 MCL cells and normal lymphocytes using an anti-nucleolin antibody. An MCL-specific interaction was observed, the presence of nucleolin being testified at several locations within and around the *Ccnd1* gene, including the above-described LR1 sites (supplemental Figure 2A-B). A higher nucleolin content was additionally observed near the breakpoint region on the rearranged chromosome 14, but not on a chromosome 8 region used as control (supplemental Figure 2C-D; supplemental Table 1). The ChIP-on-chip experiments also revealed vast amounts of lysine 9-acetylated histone H3 at the *Ccnd1* promoter (supplemental Figure 2A-B), consistent with the high transcriptional activity of this gene. Together, these data are in agreement with the previously reported presence of active chromatin marks at the *Ccnd1* promoter region in MCL cells[4] and further indicate that the relocalization of the *CCND1* locus favors its increased interaction with nucleolin.

A transactivating role for nucleolin in MCL and other non-Hodgkin B-cell lymphomas

Thus, in MCL cells, the translocated *Ccnd1* allele gets localized in a perinucleolar territory and becomes transcriptionally active, presumably under the influence of nucleolin. To further circumstantiate a transactivating role for this protein, HeLa cells were transfected with a green fluorescent protein (GFP)-nucleolin construct. GFP-positive cells were sorted and found to contain nucleolin spread throughout the nucleus (data not shown). In these transfectants, *Ccnd1* transcription levels were more than 2-fold higher than those measured in mock-transfected cells, with actin B or rRNA 18S transcription

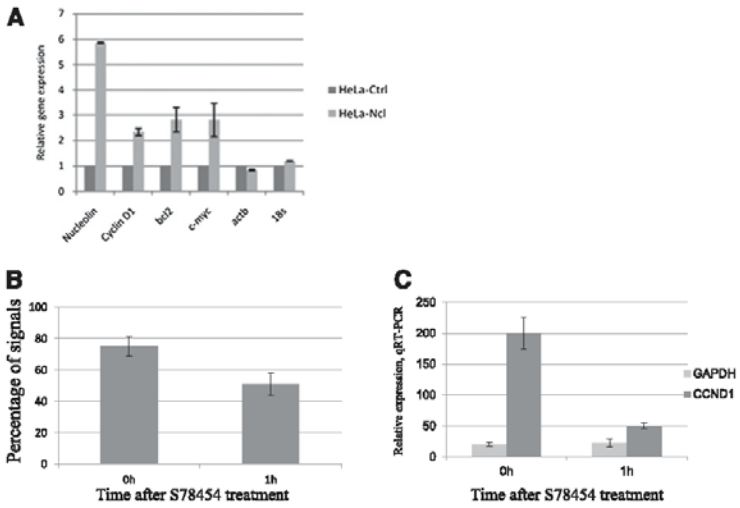

Figure 6. *Ccnd1* transcription levels and colocalization of nucleoli and *Ccnd1*. (A) A nucleolin-GFP pEGFPC1. Nucelolin construct was transfected into HeLa cells followed by selection of GFP-positive cells by flow cytofluorimetry. Transcription of nucleolin, *Ccnd1*, *c-myc*, *Bcl2*, Actin B, and 18S rRNA studied by quantitative reverse-transcription PCR in GFP-nucleolin and mock-transfected cells. Error bars represent the standard deviation between duplicates. (B-C) *Ccnd1* localization and expression in Granta-519 cells treated with a histone deacetylase inhibitor. The localization of a *Ccnd1* allele <1 μm from a nucleolus (B) as determined using 3D-FISH and *Ccnd1* transcription rate (C) as measured by quantitative reverse-transcription PCR. The data represent the average results from 2 independent experiments.

remaining unchanged (Figure 6A). Of note, a nucleolin knockdown performed in MCL cells leads to massive apoptosis, precluding any relevant experiment.[25] Thus, although highly likely, an essential role of nucleolin for Ccnd1 expression in MCL cells cannot be formally proven.

Whether the proximity with a nucleolus is important for *Ccnd1* transcription in MCL cells was further tested when Granta-519 MCL cells were submitted to Abexinostat (S78454 / PCI-24781), a pan-histone deacetylase inhibitor whose action provokes an overall disorganization of the nuclear structure. In cells thus treated, the percentage of the der14-carried *Ccnd1* alleles still localized next to a nucleolus decreased from 75% to 53% as early as 1 hour after treatment (Figure 6B), and this was accompanied by a 4-fold reduction in *Ccnd1* transcription (Figure 6C), consistent with *Ccnd1* expression in MCL cells requiring an unperturbed chromatin and nuclear organization.

Bcl2 and *c-myc*, the 2 proto-oncogenes activated in follicular and BLs, respectively, were also found activated in nucleolin transfectants (Figure 6A). Confirming the previous report that the nucleolin-containing transcription factor LR1 is a transcriptional activator for *c-myc*,[26] this further suggested that in these B-cell tumors with a t(14;18) or t(8;14) translocation, an important step in the transformation process could relate to the relocalization in the vicinity of a nucleolus of a fragment of the rearranged *IgH*-carrying chromosome. This hypothesis was tackled using the endemic BL-derived Raji and P3HR1 cell lines that harbor the canonical t(8;14)(q24;q32) translocation. In this cell line, one allele of the *c-myc* oncogene, normally on chromosome 8, gets localized immediately telomeric to an *IgH* switch region on the der14 following the translocation event. Our 3D-FISH results generated using specifically designed probes (Figure 7A; supplemental Figure 1) were very similar to those observed in MCL cells. Indeed, the *IgH* and *c-myc* alleles juxtaposed on the der14 were observed farther from the nuclear membrane than their counterparts on intact chromosomes 14 and 8 (34% vs 51% and 35% vs 54% centrally localized *IgH* and *c-myc* alleles, respectively; $P < .01$) (Figure 7B). Furthermore, the *IgH* and *c-myc* alleles on the rearranged chromosome 14 lay closer to a nucleolus than those on intact chromosomes 14 and 8 (*IgH*, 66% vs 38%, and *c-myc*, 67% vs 49% of the alleles on the rearranged vs nonrearranged chromosomes, respectively; $P < .01$) (Figure 7C).

Discussion

Experiments performed in the early and mid-1980s led to the idea that the translocation of a proto-oncogene next to a transcriptional activator would trigger molecular events crucial for cell transformation. Experiments with the *c-myc* proto-oncogene were particularly thought-provoking. Mice rendered transgenic with an unmodified *c-myc* placed immediately downstream of the *IgH* locus Eμ enhancer exhibited enlarged B-cell compartments resulting in the constant emergence of monoclonal B-cell proliferations.[27] At the time, the constructs had been designed upon the naturally occurring rearrangements observed in mouse plasmacytomas. In those early transgenic experiments, the genetic elements introduced in murine recipients were separated by hundreds of nucleotides only, far different from the genomic organization observed in human translocation-bearing B-cell tumors where the distance between the *IgH* locus and the translocated proto-oncogene can be orders of magnitude larger. Such is indeed the case in the vast majority of FLs with the t(14;18) translocation. Following the chromosomal rearrangement, one allele of the *Bcl2* antiapoptotic gene is displaced from chromosome 18 to the der14, where it now lays hundreds of kilobases telomeric to the *IgH* locus. Using the chromosome conformation capture methodology, Duan et al[28] have evidenced a direct interaction between enhancers present in the 3′ region of the *IgH* locus and the 5′ promoter region of the newly transcribed translocated *Bcl2* allele. This is in accordance with our improved understanding of chromatin loops and nuclear organization that allow regulatory elements positioned in *cis* at distances up to the megabase range to come in direct contact with each other in the 3-dimensional space of the cell nucleus (for review see Hou and Corces[29]).

Here, we have focused on MCL, a B-cell proliferation that has been less studied so far but whose chromosomal rearrangement also interests the *IgH* locus. Similar to *Bcl2* in FL, the translocated *Ccnd1* allele lies at a relatively large linear distance from transcriptional enhancers in the *IgH* locus. Also similarly, the translocation results in the expression of a gene that is silent in normal naive B cells and whose expression appears directly related to the transformation process. Importantly, MCL differs from the BL model, as transgenic

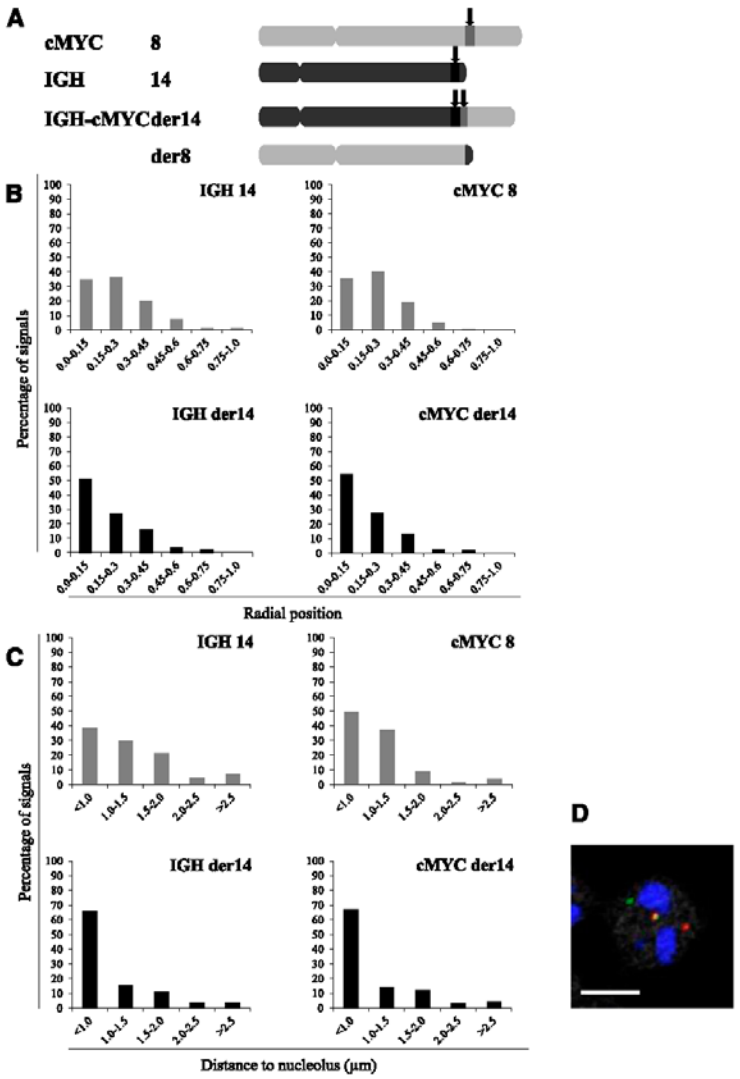

Figure 7. Radial localization and distances to closest nucleolus for *IgH* and *c-myc* loci in the Raji BL cell line. (A) Schematic representation of the chromosomes involved in the t(8;14) translocation in the Raji BL cell line and localization of the *IgH* and *c-myc* probes used. (B) Distribution of the translocated (*IgH – c-myc* der14) and nontranslocated (*IgH* 14 and *c-myc* 8) *IgH* and *c-myc* alleles according to their radial positioning in Raji cell nuclei. Distances were measured as described in Materials and methods. Histograms represent the percentage of FISH signals in the nuclear space with the range 0.0 to 0.15 corresponding to the centermost fraction of the nucleus and 0.75 to 1 to its periphery. Each graph represents a minimum of 115 FISH signals. (C) Perinucleolar relocalization of the der14 *IgH* and *c-myc* alleles in the BL Raji cell line. The *IgH* and *c-myc* alleles and nucleoli were revealed by immuno-FISH. Distances to the closest nucleolus were measured as described in Materials and methods for a minimum of 115 FISH signals. (D) A representative image of double-labeled DNA FISH in RAJI cell line. *IgH* is green, *c-myc* is red, and nucleolus (B23) is blue. Scale bar represents 5 µm.

mice expressing the *Ccnd1* gene under the control of the *IgH* Eµ enhancer do not develop lymphomas.[30] To better understand the underlying mechanisms induced by the t(11;14) translocation, we have explored the nuclear positioning of each allele of the *Ccnd1* gene in MCL cells. In both cell lines and fresh tumor cells, the translocated *Ccnd1* allele was observed farther from the nuclear membrane than the allele on the intact chromosome 11 and also farther than the usual *Ccnd1* radial position in naive B cells (data not shown). This translocation-associated displacement away from the nuclear membrane resulted in a new positioning in the vicinity of a nucleolus, a difference that was statistically significant between the *Ccnd1* alleles on the rearranged vs intact chromosomes. In MCL cells, *Ccnd1* is known to be monoallelically expressed,[20] a situation also reported for other somatic genes.[31] Our data are totally consistent with transcription taking place solely from the translocated *Ccnd1* allele.

Using immuno-FISH experiments, the *IgH* and *Ccnd1* loci present on the der14 were revealed as juxtaposed signals. In most instances, those were located in the immediate vicinity of clusters of transcriptionally active PolII molecules, in sharp contrast with the untranslocated *Ccnd1* alleles whose vast majority lay away from transcription factories. The presence of active PolII clusters in the periphery of nucleoli, which in normal cells is mostly occupied by heterochromatin and therefore not prone to PolII transcription, should not come as a surprise, because active chromatin marks have been reported previously in the *Ccnd1* promoter region of MCL cells.[4]

What triggers *Ccnd1* transcription from the der14? To answer this question, we hypothesized that a nucleolus-derived factor could contribute to this transcriptional activation. LR1 is a DNA-binding heterodimer composed of HnRNP-D and nucleolin, a major component in nucleoli and a potent transcriptional activator. Binding

sites for LR1 were identified within the *Ccnd1* gene and indeed shown to function as transcriptional enhancers. In ChIP experiments, nucleolin was directly detected in vivo within and around the *Ccnd1* gene. Furthermore, *Ccnd1* transcription was also activated in transfected HeLa cells overexpressing nucleolin. Conversely, when the overall organization of MCL Granta-519 cells was perturbed following treatment with an inhibitor of histone deacetylases, the percentage of *Ccnd1* alleles associated with a nucleolus rapidly decreased in parallel with levels of *Ccnd1* transcription. Together, these results strongly suggest that *Ccnd1* transcription gets activated in MCL cells, because the translocated *Ccnd1* allele gets localized in the nucleolin-abundant environment found in perinucleolar areas. In this regard, it is tempting to speculate that nucleolin and possibly other nucleolar components such as nucleophosmin or ribosomal proteins could also participate in PolII transcription in malignant cells. Interestingly, nucleophosmin associates with another factor, CTCF.[32] We have observed MCL-specific binding of CTCF in the vicinity of the *Ccnd1* promoter. Similar binding has been previously reported in BL cells.[33] This binding may play a role both in the insulation of the *Ccnd1* promoter and in its transcriptional regulation via CTCF.

The literature contains one report of inhibition of nucleolin expression in HeLa cells and fibroblasts treated for 5 days with a combination of small interfering RNA. This inhibition was accompanied by many abnormalities, including a lowered proliferation index, an accumulation of cells in the G2 phase of the cell cycle, and eventual entry of the cells into apoptosis.[25] In addition to its ribosomal-related functions, nucleolin has also been characterized as a histone chaperone and chromatin co-remodeler. In view of its variable cellular localization and pleiotropic functions and because of the well-known difficulties for efficiently transfecting B lymphocytes, we have not attempted here to extinguish nucleolin expression in MCL cells.

The results we have obtained are partially contradictory to those reported by the Epner's group,[19] who found no difference in positioning between the translocated and untranslocated *Ccnd1* alleles. Possible reasons for this discrepancy include our methodology to measure radial positioning and distances to a nucleolus. Here, we have carefully distinguished between the translocated and nontranslocated alleles, the latter being almost never observed in the proximity of a nucleolus. Also, the 3D-FISH methodology we have applied to MCL cell lines and malignant cells from MCL patients may have allowed more precise measurements than in the 2-dimensional FISH experiments performed by Liu et al.[19]

To account for *Ccnd1* expression in MCL cells, we propose a model whereby the key event occurs when one *Ccnd1* allele gets newly localized to a transcriptionally favorable perinucleolar territory in these cancer cells. Can a similar mechanism apply in other hematopoietic malignancies with chromosomal rearrangements interesting acrocentric chromosomes? In the major reciprocal t(8;14) translocation associated with Burkitt lymphomas, the chromosome 8-carried proto-oncogene *c-myc* gets localized head to head next to the *IgH* locus on the rearranged acrocentric 14. Differing from sporadic forms of BL where the 5′ end of *c-myc* is decapitated following the chromosomal break, in the endemic form, an intact *c-myc* gene gets juxtaposed to the *IgH* locus along with hundreds of kilobases of genomic sequence from the 5′ region of *c-myc*. In this new setting, *c-myc* is localized at a long distance from the *IgH* locus, reminiscent of the situation in FL and MCL. In Raji, a prototypical endemic BL cell line with *c-myc* hundreds of kilobases telomeric to the *IgH* locus, our experiments have revealed that the rearranged chromosome 14 fragment, which now carries *IgH*, and *c-myc* gets repositioned close to a nucleolus. In this new setting, *c-myc* may fall in the action range of nucleolar factors such as nucleolin, which in our transcription assays was found to activate *c-myc* even better than *Ccnd1*. Further experiments will be necessary to explore the actual relevance of such a mechanism in various B-cell proliferations and to investigate the respective role of nucleolar components and *cis* regulation in the activation of genes crucial for transformation in MCL, BL, and other non-Hodgkin B-cell lymphomas.

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Authorship

Contribution: J.A., A.P., O.I., M.K., E.Z.-Z., D.M., and N.P. performed research; A.B., J.W., M.L., V.R., O.I., S.V.R., M.L., and Y.S.V. analyzed data; V.C.-C. and V.R. contributed reagents; and M.L. and Y.S.V. wrote the paper

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References

1. Küppers R. Mechanisms of B-cell lymphoma pathogenesis. *Nat Rev Cancer*. 2005;5(4):251-262.
2. Klein IA, Resch W, Jankovic M, et al. Translocation-capture sequencing reveals the extent and nature of chromosomal rearrangements in B lymphocytes. *Cell*. 2011;147(1):95-106.
3. Jares P, Colomer D, Campo E. Molecular pathogenesis of mantle cell lymphoma. *J Clin Invest*. 2012;122(10):3416-3423.
4. Liu H, Wang J, Epner EM. Cyclin D1 activation in B-cell malignancy: association with changes in histone acetylation, DNA methylation, and RNA polymerase II binding to both promoter and distal sequences. *Blood*. 2004;104(8):2505-2513.
5. Degan M, Doliana R, Gloghini A, et al. A novel bcl-1/JH breakpoint from a patient affected by mantle cell lymphoma extends the major translocation cluster. *J Pathol*. 2002;197(2):256-263.
6. Greisman HA, Lu Z, Tsai AG, Greiner TC, Yi HS, Lieber MR. IgH partner breakpoint sequences provide evidence that AID initiates t(11;14) and t(8;14) chromosomal breaks in mantle cell and Burkitt lymphomas. *Blood*. 2012;120(14):2864-2867.
7. Cremer M, von Hase J, Volm T, et al. Non-random radial higher-order chromatin arrangements in nuclei of diploid human cells. *Chromosome Res*. 2001;9(7):541-567.
8. Cremer T, Cremer M, Dietzel S, Müller S, Solovei I, Fakan S. Chromosome territories—a functional nuclear landscape. *Curr Opin Cell Biol*. 2006;18(3):307-316.
9. Solovei I, Kreysing M, Lanctôt C, et al. Nuclear architecture of rod photoreceptor cells adapts to vision in mammalian evolution. *Cell*. 2009;137(2):356-368.
10. Croft JA, Bridger JM, Boyle S, Perry P, Teague P, Bickmore WA. Differences in the localization and morphology of chromosomes in the human nucleus. *J Cell Biol*. 1999;145(6):1119-1131.

11. Küpper K, Kölbl A, Biener D, et al. Radial chromatin positioning is shaped by local gene density, not by gene expression. *Chromosoma*. 2007;116(3):285-306.
12. Mahy NL, Perry PE, Gilchrist S, Baldock RA, Bickmore WA. Spatial organization of active and inactive genes and noncoding DNA within chromosome territories. *J Cell Biol*. 2002;157(4): 579-589.
13. Petrov AV, Allinne J, Pirozhkova IV, Laoudj D, Lipinski M, Vassetzky YS. A nuclear matrix attachment site in the 4q35 locus has an enhancer-blocking activity in vivo: implications for the facio-scapulo-humeral dystrophy. *Genome Res*. 2008;18(1):39-45.
14. Solovei I, Walter J, Cremer M, Habermann FA, Schermelleh L, Cremer T. FISH: a practical approach. In: Squire J, Beatty B, Mai S, eds. *FISH: A Practical Approach*. Oxford: Oxford Univ Press; 2002.
15. Pichugin A, Le Bourhis D, Adenot P, et al. Dynamics of constitutive heterochromatin: two contrasted kinetics of genome restructuring in early cloned bovine embryos. *Reproduction*. 2010;139(1):129-137.
16. Pichugin A, Beaujean N, Vignon X, Vassetzky Y. Ring-like distribution of constitutive heterochromatin in bovine senescent cells. *PLoS ONE*. 2011;6(11):e26844.
17. Aguirre-Lavin T, Adenot P, Bonnet-Garnier A, et al. 3D-FISH analysis of embryonic nuclei in mouse highlights several abrupt changes of nuclear organization during preimplantation development. *BMC Dev Biol*. 2012;12:30.
18. Kim K, Dimitrova DD, Carta KM, Saxena A, Daras M, Borowiec JA. Novel checkpoint response to genotoxic stress mediated by nucleolin-replication protein a complex formation. *Mol Cell Biol*. 2005; 25(6):2463-2474.
19. Liu H, Huang J, Wang J, et al. Transvection mediated by the translocated cyclin D1 locus in mantle cell lymphoma. *J Exp Med*. 2008;205(8): 1843-1858.
20. Hosokawa Y, Arnold A. Mechanism of cyclin D1 (CCND1, PRAD1) overexpression in human cancer cells: analysis of allele-specific expression. *Genes Chromosomes Cancer*. 1998; 22(1):66-71.
21. Wallace JA, Felsenfeld G. We gather together: insulators and genome organization. *Curr Opin Genet Dev*. 2007;17(5):400-407.
22. Wingender E, Dietze P, Karas H, Knüppel R. TRANSFAC: a database on transcription factors and their DNA binding sites. *Nucleic Acids Res*. 1996;24(1):238-241.
23. Hanakahi LA, Dempsey LA, Li MJ, Maizels N. Nucleolin is one component of the B cell-specific transcription factor and switch region binding protein, LR1. *Proc Natl Acad Sci USA*. 1997; 94(8):3605-3610.
24. Hanakahi LA, Maizels N. Transcriptional activation by LR1 at the Emu enhancer and switch region sites. *Nucleic Acids Res*. 2000;28(14): 2651-2657.
25. Ugrinova I, Monier K, Ivaldi C, et al. Inactivation of nucleolin leads to nucleolar disruption, cell cycle arrest and defects in centrosome duplication. *BMC Mol Biol*. 2007;8:66.
26. Brys A, Maizels N. LR1 regulates c-myc transcription in B-cell lymphomas. *Proc Natl Acad Sci USA*. 1994;91(11):4915-4919.
27. Adams JM, Harris AW, Pinkert CA, et al. The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. *Nature*. 1985;318(6046): 533-538.
28. Duan H, Xiang H, Ma L, Boxer LM. Functional long-range interactions of the IgH 3′ enhancers with the bcl-2 promoter region in t(14;18) lymphoma cells. *Oncogene*. 2008;27(53): 6720-6728.
29. Hou C, Corces VG. Throwing transcription for a loop: expression of the genome in the 3D nucleus. *Chromosoma*. 2012;121(2):107-116.
30. Fiancette R, Amin R, Truffinet V, et al. A myeloma translocation-like model associating CCND1 with the immunoglobulin heavy-chain locus 3′ enhancers does not promote by itself B-cell malignancies. *Leuk Res*. 2010;34(8):1043-1051.
31. Takizawa T, Gudla PR, Guo L, Lockett S, Misteli T. Allele-specific nuclear positioning of the monoallelically expressed astrocyte marker GFAP. *Genes Dev*. 2008;22(4):489-498.
32. Yusufzai TM, Tagami H, Nakatani Y, Felsenfeld G. CTCF tethers an insulator to subnuclear sites, suggesting shared insulator mechanisms across species. *Mol Cell*. 2004;13(2):291-298.
33. Gombert WM, Farris SD, Rubio ED, Morey-Rosler KM, Schubach WH, Krumm A. The c-myc insulator element and matrix attachment regions define the c-myc chromosomal domain. *Mol Cell Biol*. 2003;23(24):9338-9348.
34. Bell AC, West AG, Felsenfeld G. The protein CTCF is required for the enhancer blocking activity of vertebrate insulators. *Cell*. 1999;98(3): 387-396.

DNA polymorphism and epigenetic marks modulate the affinity of a scaffold/matrix attachment region to the nuclear matrix.

Kisseljova NP, Dmitriev P, Katargin A, Kim E, Ezerina D, Markozashvili D, Malysheva D, Planche E, Lemmers RJ, van der Maarel SM, Laoudj-Chenivesse D, Lipinski M, Vassetzky YS.
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Mechanisms that regulate attachment of the scaffold/matrix attachment regions (S/MARs) to the nuclear matrix remain largely unknown. We have studied the effect of the simple sequence length polymorphism (SSLP), DNA methylation and chromatin organization in an S/MAR implicated in a facioscapulohumeral dystrophy (FSHD), a hereditary disease linked to a partial deletion of the D4Z4 repeat array on chromosome 4q. This FSHD-related nuclear matrix attachment region (FR-MAR) loses its efficiency in myoblasts from FSHD patients.

First, we have shown that DNA-methylation level of FR-MAR affects its efficiency of attaching to the nuclear matrix (NM). Moreover, DNA with the 8nt+ SSLP haplotype had a much higher affinity to the NM than the 8nt- haplotypes (for the details refer to the original article). Then we wondered whether other features of chromatin structure could further contribute to this association.

My role in the paper was to perform a ChIP analysis of the FR-MAR region of normal and FSHD myoblasts with antibodies against Methyl-CpG-binding protein (MeCP2), and histone acetylated on Lys9 (H3K9ac). DNA was isolated from immunoprecipitates and quantified via TaqMan qPCR using G17-F/G17-R primers. We have observed that there were no H3K9ac in FR-MAR region in normal myoblasts, contrasting with FSHD cell lines. MeCP2, in opposite, was present in high levels within the FR-MAR region in normal myoblasts and decreased in

FSHD (Figure 3 in the article). Interestingly, the presence of MeCP2 appeared to be inversely related to that of H3K9ac not only in primary myoblasts but also in carcinoma cell lines, suggesting that it indicates a common mechanism of MeCP2/chromatin binding operates in two types of cells (Supplementary Figure S2 in the article).

To further explore these possible relationships between various features of the chromatin structure and FR-MAR affinity to the NM, we treated the human cervical carcinoma cells CaSki with the methylation inhibitor 5-aza-dC, the histone deacetylation inhibitor trichostatin A or a combination there of. Isolation of NM and further qPCR have shown that the FR-MAR attachment to the NM depends on both the methylation of the DNA and the acetylation levels in the chromatin-contained histones.

Thus, three criteria were found to be important for high-affinity interaction between the FR-MAR and the nuclear matrix: the presence of a specific SSLP haplotype in chromosomal DNA, the methylation of one specific CpG within the FR-MAR and the absence of H3K9ac in the relevant chromatin fragment.

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ARTICLE

DNA polymorphism and epigenetic marks modulate the affinity of a scaffold/matrix attachment region to the nuclear matrix

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Mechanisms that regulate attachment of the scaffold/matrix attachment regions (S/MARs) to the nuclear matrix remain largely unknown. We have studied the effect of simple sequence length polymorphism (SSLP), DNA methylation and chromatin organization in an S/MAR implicated in facioscapulohumeral dystrophy (FSHD), a hereditary disease linked to a partial deletion of the D4Z4 repeat array on chromosome 4q. This FSHD-related nuclear matrix attachment region (FR-MAR) loses its efficiency in myoblasts from FSHD patients. Three criteria were found to be important for high-affinity interaction between the FR-MAR and the nuclear matrix: the presence of a specific SSLP haplotype in chromosomal DNA, the methylation of one specific CpG within the FR-MAR and the absence of histone H3 acetylated on lysine 9 in the relevant chromatin fragment.

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Keywords: nuclear matrix; SSLP; facioscapulohumeral dystrophy; epigenetics; cancer cells

INTRODUCTION

Scaffold/matrix attachment regions (S/MARs) are specialized genomic DNA sequences that exhibit high affinity to the nuclear matrix (NM) *in vitro* and *in vivo*. S/MARs often colocalize with or are situated in close proximity to specific sequences, including replication origins, insulators and enhancers (for a review see Vassetzky *et al*[1] and Razin *et al*[2]). S/MARs anchor chromatin onto the NM, thereby organizing the genomic DNA into topologically distinct loop domains that are important for the regulation of replication and transcription.[2]

Despite numerous genomic studies,[3–7] no consensus S/MAR sequence has been identified so far.[8] S/MARs can be both A/T[9] or G/C rich.[10] Moreover, the strength and specificity of S/MAR attachment to the NM varies during ontogenesis,[11] in cancer[12] and various genetic diseases such as facioscapulohumeral dystrophy (FSHD).[13,14] Several factors potentially affect the association of DNA with the NM. These include the DNA sequence itself and its epigenetic state.[15–18]

We have recently characterized an S/MAR within the subtelomeric region of chromosome 4q.[13,14] This region (Figure 1a) has a complex structure and includes the D4Z4 macrosatellite repeat array, which consists of 3.3 kb repeated units with a number of units varying between individuals.[19] This S/MAR, designated as FR-MAR for FSHD-related matrix attachment region,[13] is located centromerically to the D4Z4 array and includes a simple sequence length polymorphism (SSLP) (for a review see Dmitriev *et al*[20]). The 4q subtelomeric region is subject to extensive epigenetic changes between normal and pathological cells. Thus, we have shown that in FSHD the FR-MAR is partially detached from the NM.[13,14] The mechanisms underlying the observed interaction between the FR-MAR and the NM remain largely unknown. Several factors could have a role. First, the protein composition of the NM could vary between normal and pathological tissues. For example, levels of the NM protein vimentin were found to differ between FSHD and normal myoblasts.[21] Also, the nucleotide sequence in the FR-MAR region could affect the affinity for the NM. Indeed, several SSLPs have been identified that are specifically associated with FSHD.[22–24] Finally, the chromatin structure and DNA methylation level could also modify the FR-MAR binding to the NM. Other genetic and epigenetic abnormalities affecting the subtelomeric region of chromosome 4q have also been observed in various pathologies including the Rett and ICF syndromes,[25] as well as in several types of cancer.[26–32]

Here, we have tested whether mechanisms controlling the association of the FR-MAR to the NM could be similar in different cell types. We have used primary myoblasts from FSHD patients and cervical carcinoma cell lines where the D4Z4 repeat array can be either strongly methylated or demethylated.[30] From our experiments, the FR-MAR binds the NM depending upon a combination of SSLP and chromatin modifications including DNA CpG methylation, histone H3 modifications and MeCP2 binding.

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MATERIALS AND METHODS

Cell lines

The human cervical carcinoma cells CaSki and C-33A (American Type Culture Collection, Manassas, VA, USA) were maintained in DMEM supplemented with 5–10% FCS. Primary human myoblasts were isolated and differentiated as described.[33] The primary human myoblasts are described in Table 1. Cells were treated with DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (5-aza-dC; Sigma, St Louis, MO, USA) for 3 days. The medium containing 5 μM 5-aza-dC was changed daily. Cells were treated with trichostatin A (Sigma) at a concentration of 1 μM for 1 day before harvesting.

Analysis of SSLP

DNAs (total and associated with NM) were PCR amplified using oligonucleotide primers: SSLP-F, 5′-GCTCTTGAGCTCGTCTTGGACA-3′ and SSLP-R, 5′-CTTCAGAGGCATTTGGCAGAAG-3′. The PCR products were cloned into pGEM-T Easy vector (Promega, Madison, WI, USA), 65–100 clones were used for automated sequencing ('Genome' Center, Moscow, Russia; Millegen, Toulouse, France), analyzed and the quantity of clones with or without eight-nucleotide (8nt) insert was calculated.

Nuclei and nuclear matrices

Nuclei were isolated from the cell lines essentially as described elsewhere.[34] Nuclear matrices were prepared by treatment of the isolated nuclei with NaCl as follows: digestion buffer (100 mM NaCl, 25 mM KCl, 10 mM Tris-HCl at pH 7.5, 0.25 mM spermidine) was added to 10^7 nuclei to a final volume of 400 μl. DNase I was added to a final concentration of 100 μg/ml, and the samples were digested for 2 h at 4 °C, followed by the addition of $CuCl_2$ to a final concentration of 1 mM for 10 min at 4 °C. The nuclei were then extracted by the addition of one volume of a buffer containing 4 M NaCl, 20 mM EDTA and 40 mM Tris-HCl at pH 7.5. The resulting nuclear matrices were spun in a microfuge at 2000 *g* for10 min at 4 °C and then washed three times with a buffer containing 2 M NaCl, 10 mM EDTA and 20 mM Tris-HCl at pH 7.5. Nuclear matrices were digested with proteinase K and DNA was extracted with phenol–chloroform. The medium molecular weight of the MAR fraction was ~400 bp. Total DNA was prepared from the intact nuclei by proteinase K digestion, followed by phenol–chloroform extraction. Two or three independent experiments were carried out in each case and the data were either averaged or pooled.

Table 1 Primary human myoblasts used in the study

Type	Patient	Norm/ FSHD	Tissue	Age (years)	Sex	Number of D4Z4 repeats	Reference
MB	N047	Norm	Quadriceps	43	M	ND	21
MB	N042	Norm	Quadriceps	24	F	ND	21
MB	M044	FSHD 1	Pyramidal	54	F	5/7	21
MB	M047	FSHD 1	Quadriceps	38	F	7	21
MB	M054	FSHD 1	Quadriceps	25	M	4	21
MB	M043	FSHD 2	Infraspinatus	41	M	ND	21

Abbreviations: MB, myoblasts; ND, not determined.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed on 5×10^6 cells fixed with 1% formaldehyde using the ChIP it express kit with protein G magnetic beads (Active Motif, Carlsbad, CA, USA) according to the manufacturer's protocol. Chromatin was sheared by sonication and precipitated with MeCP2 antibodies (chip grade, ab2828; Abcam, Cambridge, UK) or anti-histone H3 acetylated on lysine 9 (H3K9ac) antibodies (ab4441; Abcam) according to the manufacturer's protocol. After the removal of formaldehyde crosslinks, MeCP2-precipitated chromatin were treated with proteinase K (0.5 mg/ml) and used in qPCR amplification via primers: G17-F, 5′-GGAAC GACCCTTCTCAGACAGTA-3′ and G17-R, 5′-GCCTAAAGTTGAAAAC TAAAATCACACATGA-3′ together with the TaqMan probe G17: 5′-FAM-CACCCTGCCAACTATT-3′ and analyzed using ABIPrism 7900 real-time PCR device (Applied Biosystems, Saint Aubin, France). Alternatively, MeCP2 antibody-immunoprecipitated DNA was used in bisulfite conversion reactions and sequencing.

Bisulfite-based cytosine methylation analysis

The bisulfite conversion reaction was carried out using an EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions under the following conditions: 15 cycles (30 s at 95 °C and 15 min at 50 °C). Total DNAs digested overnight with *Bam*HI and *Hind*III restriction endonucleases or with *Hind*III only (Fermentas, Vilnius, Lithuania) to improve the bisulfite conversion, while the NM DNAs and immunoprecipitated DNAs were used without preliminary digestion. Then, the converted DNA samples were used for the PCR amplification by Maxima Hot StartTaq DNA polymerase according to the manufacturer's instructions (Fermentas). Two sets of FR-MAR-specific primers were used. To analyze the methylation status of CpG_1–CpG_4, we used a set of primers corresponding to the upper strand of bisulfite-converted DNA: F1*, 5′-ATTTTTAGGTGAGATG GTTTG-3′ and R1*, 5′-CCTAAAATTAAAAACTAAAATCACAC-3′. To analyze simultaneously the methylation status of CpG_2–CpG_4 and obtain the sequence of SSLP, the second set of primers corresponding to the lower strand of bisulfite-converted DNA was used: F2*, 5′-CAAACTTTAAACAATATATATAACTC-3′ and R2*, 5′-TTTTAGAGGTATTTGGTAGAAG-3′; primer positions are shown in Figure 1. The resulting PCR products were cloned into pGEM-T Easy vector (Promega) and used for automated sequencing ('Genome' Center; Millegen).

Statistical analysis

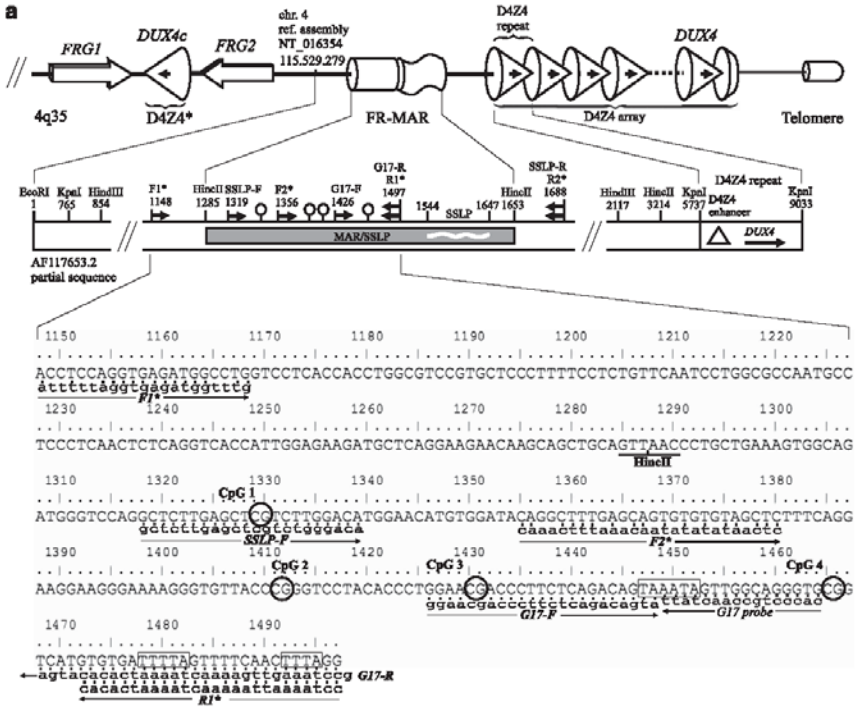
Fisher's exact test (two-tailed) and χ^2-test were used to assess the statistical significance of differences between methylation levels of FR-MAR and SSLP variation in different samples, respectively. All statistical procedures were performed using 'SATISTICA 6' software (StatSoft, Boston, MA, USA).

RESULTS

The subtelomeric region of human chromosome 4q, represented in Figure 1a, is the subject of particular attention because of its association with several pathologies, including FSHD, a highly frequent muscular dystrophy. FR-MAR is a *Hin*cII 368-bp-long DNA segment located proximally to the D4Z4 repeat array. Four CpG dinucleotides (numbered CpG_1–CpG_4) are prone to DNA methylation within the FR-MAR. All four are contained within a 348-bp-long PCR fragment amplifiable using the F1* and R1* oligonucleotides (Figure 1a). On its telomeric side, the MAR exhibits an SSLP with four major haplotypes referred to as 161, 163, 166

Figure 1 Organization of the 4q35 region and the FR-MAR. (**a**) Schematic representation of the subtelomeric region of chromosome 4 (4q35) showing *FRG1* gene, D4Z4 repeat array, isolated D4Z4 repeat homolog (D4Z4*), FR-MAR region that comprises an SSLP and its partial sequence showing major restriction sites, including *Hin*cII (GTY^RAG) that delimits the 5′ border of the FR-MAR, four CpG sites within FR-MAR (circles), PCR primers (arrowheads) and a TaqMan probe. *Primers used for amplification of a bisulfite-converted DNA. Sites required for MeCP2 binding ($[A/T]_{\geq 4}$) are framed. (**b**) Schematic representation of the FR-MAR polymorphic region and its partial sequence showing four major SSLPs: 161, 163, 166 and 168, which can be generally described by the formula $(CA)_n-(AA+/-)-(CA)_n-(G/T)-(CT)_n-(8nt+/-)$, *Hin*cII restriction site that delimits the 3′ border of the FR-MAR, PCR primers used to amplify non-converted (arrowheads) or bisulfite-converted DNA (*). Nucleotide numbering is according to AF117653.2 (GenBank), the first nucleotide of AF117653.2 corresponds to the nucleotide 115529279 of GRCh37.p10 (hg19) human genome reference assembly (NT_016354, GenBank).

and 168. An 8nt-long segment being present in haplotypes 166 and 168 but not in haplotypes 161 and 163 (Figure 1b), these are grouped as 8nt+ and 8nt−, respectively. Of note, the same haplotypes are also found in the duplicated FR-MAR present within the q26 region of human chromosome 10.[22–24] Consequently, any cell contains four copies and up to four haplotypes of the FR-MAR. All four copies of



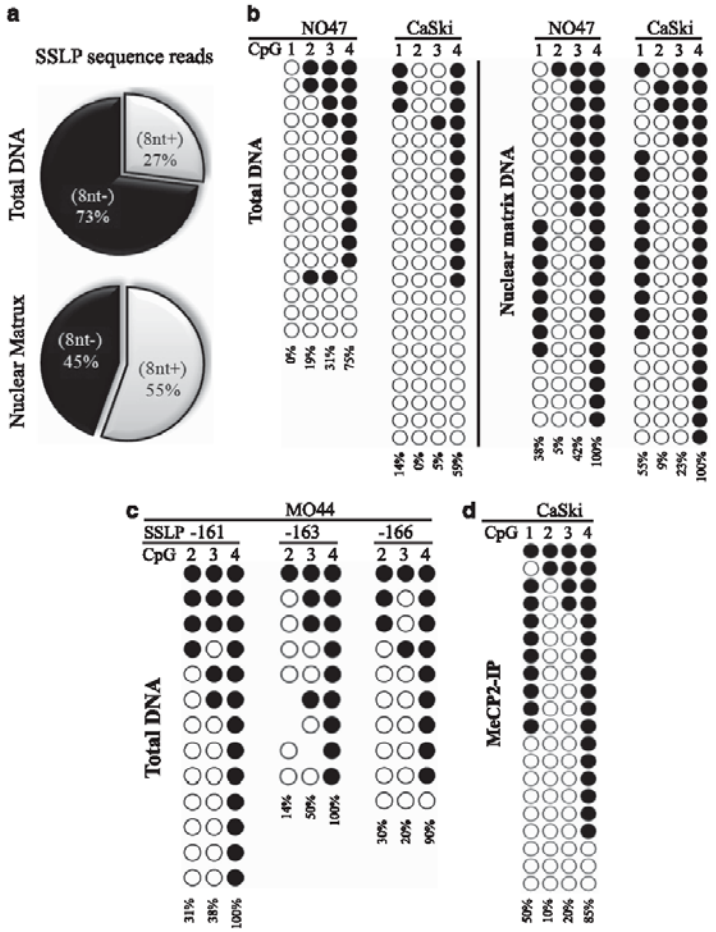


Figure 2 Effect of SSLP and DNA methylation on the NM binding. (**a**) The efficiency of FR-MAR binding *in vivo* to the NM interaction depends on the presence of the 8nt sequence in the SSLP. DNA isolated from the NM fraction or total genomic DNA was PCR amplified using SSLP-F and SSLP-R primers, cloned into pGEM-T vector and sequenced; 100 and 65 clones were analyzed for total DNA and the NM, respectively. The percentage of clones with 8nt+ and 8nt− FR-MAR variants is indicated. The data represent a sum of two independent matrix isolation experiments. (**b** and **c**) Schematic representation of results of bisulfite sequencing of total genomic DNA isolated from normal human primary myoblasts and cervical carcinoma cell line. Sodium bisulfite treatment of DNA followed by PCR amplification (primers F1* and R1*; Figure 1a) converts all cytosines of the original DNA into thymines, but all 5-methylcytosines remain unchanged. After this procedure, PCR products were cloned and sequenced: each row of circles represents a cloned individual DNA molecule; closed circles – methylated CpG, open circles – unmethylated CpG and uncharacterized CpGs are represented as gaps. Numbers above the panels correspond to $CpG_{1\ 4}$ within FR-MAR from 5′ to 3′ end. The percentage of methylation is shown under each sample. (**b**) Total genomic and NM-associated DNA was isolated from normal human myoblasts NO47 and carcinoma cell line CaSki bisulfite-converted and PCR-amplified using primers F1* and R1*, cloned and sequenced. (**c**) There are no clear differences between methylation statuses of three FR-MAR variants. Total genomic DNA was isolated from FSHD myoblasts MO44 with three SSLPs, 161,163,166,[24] and bisulfite-converted and PCR-amplified using primers F2* and R2*, cloned and sequenced. The methylation status of $CpG_{2\ 4}$ is represented. (**d**) DNA isolated from CaSki chromatin immunoprecipitated with MeCP2-specific antibody (IP).

FR-MAR are attached to the NM in normal primary human myoblasts, as was previously demonstrated by *in situ* fluorescent hybridization on nuclear halos.[13]

Preferential association of 8nt+ SSLP haplotypes with the NM

We have tested whether the presence of the 8nt sequence impacted the efficiency of FR-MAR attachment to the NM. For this purpose, we have used a normal myoblast cell line, NO47, which contains three of the most frequent polymorphisms: 161 and 163, both 8nt−, and 166, 8nt+. In this cell line, the three haplotypes are present in a 2:1:1 ratio, respectively. Total and NM-associated DNA were isolated, the SSLP region of the FR-MAR was PCR amplified and cloned. The relative abundance of 8nt+ and 8nt− haplotypes was then compared between clones derived from total vs NM-attached DNA. As expected, approximately one-fourth of the clones isolated from total DNA (27 clones) had an 8nt+ status *vs* almost 75% being 8nt− (73 clones) (Figure 2a, left panel). Strikingly, the latter percentage fell to 45% of clones derived from NM-associated fraction (29 clones), with the percentage of 8nt+ clones increasing twofold to 55% (36 clones) (Figure 2a, $P<10^{-3}$). These findings suggest that DNA with

the 8nt+ SSLP haplotype had a much higher affinity to the NM than the 8nt− haplotypes (Figure 2a, right panel).

Methylated FR-MAR sequences are associated with NM

We then asked whether this differential binding of the FR-MAR sequence to the NM could be dependent on the methylation status in this DNA region. It is known that the methylation level of the D4Z4 array varies between normal cells, FSHD myoblasts and cervical carcinoma cells.[19,29–32] However, the methylation status of the FR-MAR sequence was not studied earlier. The total DNA was isolated from several muscular and cervical cell lines and treated as described in Materials and methods section. Seventy-five to 100% of the clones obtained from normal and FSHD myoblasts exhibited a methylated CpG_4, with the other three CpGs being methylated to a lower extent. A strong demethylation of four CpGs was revealed in the cervical carcinomas compared with normal cervix tissues ($P<0.001$; Supplementary Figure S1). We then isolated NM-associated DNA from two cell lines, NO47, a primary myoblast cell line, and CaSki, a cervical carcinoma cell line. The total DNA from these two cell lines contained a mixture of methylated and unmethylated CpG within the FR-MAR. In both cases, the clones obtained from the NM-associated fraction were more frequently methylated in each of the four CpGs than those produced from total DNA. Of the four CpGs, CpG_4 remained the most frequently methylated with 100% of the clones methylated in both cell lines (Figure 2b). Interestingly, when tested *in vitro*, an unmethylated FR-MAR was unable to bind NM (data not shown). Taken together, these results suggest that a high level of methylation could play a positive role in NM attachment.

We then tested the hypothesis that 8nt+ haplotypes bind the NM more efficiently than 8nt− haplotypes because of a higher methylation status of the DNA sequence. Three of the four SSLP haplotypes could be tested using the FSHD-derived myoblast cell line MO44, which contains the 161 (two alleles), 163 and 166 haplotypes. No statistically significant differences could be noticed between the DNA methylation patterns associated with each of the three haplotypes ($P>0.05$, Fisher exact test; Figure 2c). In these experiments, the methylation status of CpG_1 could not be determined as we could not obtain stable clones of PCR products that would include all four CpGs and the SSLP region. These experiments demonstrated that all FR-MAR alleles have similar methylation pattern independent of their haplotypes (8nt+ or 8nt−) or location (4q or 10q). From these data, it appears that the differential binding of the different FR-MAR haplotypes to the NM is not directly related to their DNA methylation level.

FR-MAR binds the NM and the MeCP2 protein depending upon its chromatin structure

Having found that DNA methylation affects FR-MAR attachment to the NM, we wondered whether other features of chromatin structure could further contribute to this association. We thus used ChIP to assess whether FR-MAR-containing chromatin was enriched in H3K9ac, an epigenetic mark specific of active chromatin. In normal myoblasts, no H3K9ac was identified within the FR-MAR, contrasting with FSHD cell lines (Figure 3a). The observed chromatin pattern was FR-MAR-specific as it did not spread toward the neighboring regions (Supplementary Figure S3).

A specific association of DNA with the NM also relies on matrix proteins such as the methyl-CpG-binding protein MeCP2,[35] which participates in the formation of chromatin loops.[36,37] A methylated CpG duplex flanked by $[A/T]_{\geq 4}$ motif is required for high-affinity MeCP2 binding.[38] Such a motif is indeed present within the FR-MAR,

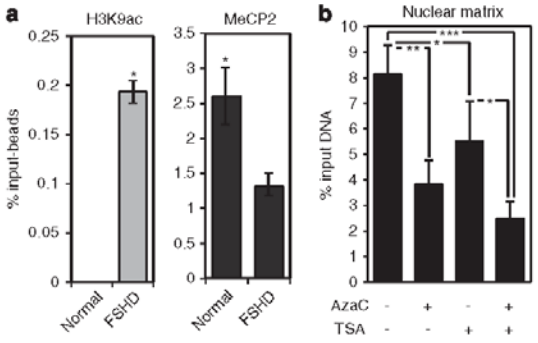


Figure 3 FR-MAR chromatin structure analysis and the effect of chromatin-modifying drugs on the affinity of the FR-MAR for the NM. (**a**) ChIP analysis of the FR-MAR region of normal and FSHD myoblasts. The chromatin was immunoprecipitated with antibodies against MeCP2, and H3K9ac, DNA was isolated from immunoprecipitates and quantified via TaqMan qPCR using G17-F/G17-R primers (Figure 1a). The results of the quantification are presented as an enrichment relative to input DNA (total chromatin) expressed in percentage after subtraction of unspecific chromatin absorption on magnetic beads; the experiments have been carried out in triplicate; **P*-value <0.05. (**b**) Chromatin-modifying drugs affect the affinity of the FR-MAR for the NM. Cells of the cervix carcinoma cell line CaSki were treated either with 5 μM DNA methyltransferase inhibitor AzaC, with 1 μM TSA or a combination of both; nuclear matrices were isolated and the amount of the FR-MAR in the input DNA and the NM fraction was quantified using qPCR. The data are presented as an enrichment relative to input DNA expressed in percentage; the average of three independent experiments is shown; **P*-value <0.05; ***P*-value <0.01; ****P*-value <0.001.

one was between CpG_3 and CpG_4 and the other after CpG_4 (Figure 1a). We have looked for the presence of MeCP2 within the FR-MAR region in normal and FSHD myoblasts. High levels of MeCP2 were detected in normal myoblasts, whereas a lower level of MeCP2 was present in FSHD myoblasts (Figure 3a). Interestingly, the presence of MeCP2 appeared to be inversely related to that of H3K9ac not only in primary myoblasts but also in carcinoma cell lines, suggesting that it indicates a common mechanism of MeCP2/chromatin binding operates in two types of cells (Supplementary Figure S2). We next analyzed the methylation status of MeCP2-associated FR-MAR in carcinoma cell line CaSki. The clones obtained from the DNA fraction immunoprecipitated by MeCP2-specific antibodies were more frequently CpG4 methylated than those produced from total DNA (Figures 2b and d). These data suggest a common mechanism of MeCP2-mediated chromatin attachment to NM in the two types of cells.

Chromatin-modifying drugs affect the FR-MAR association with the NM

To further explore these possible relationships between various features of the chromatin structure and FR-MAR affinity to the NM, we then treated CaSki cells with the methylation inhibitor 5-aza-dC, the histone deacetylation inhibitor trichostatin A (TSA) or a combination there of. Nuclear matrices were isolated from both control and drug-treated cells followed by qPCR analysis of the FR-MAR DNA. In control cells, the NM fraction contained approximately 8% of the total input FR-MAR DNA. This percentage decreased twofold in 5-aza-dC-treated cells and approximately 25% in TSA-treated cells (Figure 3b). An additive effect was obtained when

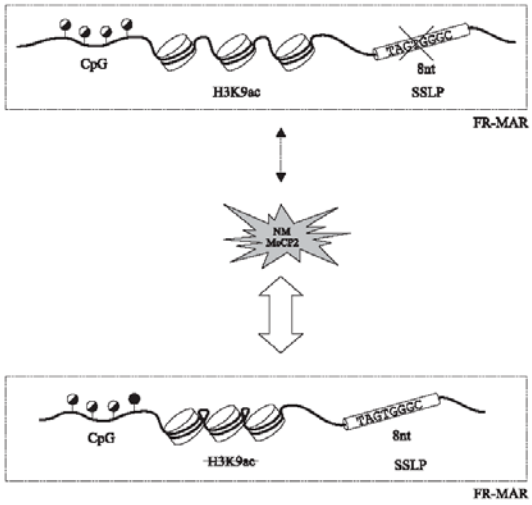


Figure 4 Three factors contribute, either independently or together, to the FR-MAR binding to the NM: DNA methylation, chromatin structure and the SSLP. Methylation of CpG_4, low level of the H3K9ac and the presence of the 8nt+ SSLP within the FR-MAR region are associated with a more efficient interaction of FR-MAR with the NM.

the two drugs were combined (Figure 3b). Taken together, these results are consistent with the notion that the FR-MAR attachment to the NM depends on both the methylation of the DNA and the acetylation levels in the chromatin-contained histones.

DISCUSSION

An important aspect of the regulation of gene expression depends upon the organization of chromosomal DNA into chromatin loops (for a review see Razin *et al*[2]). This relies upon S/MAR regions that have a major role in defining the limits of various chromatin loops. However, the molecular mechanism underlying this loop organization remains poorly characterized. Previously, we have demonstrated that the organization of chromatin loops was altered in myoblasts from patients with FSHD.[13] FSHD is also associated with a partial loss of DNA methylation and heterochromatin-specific histone modifications in D4Z4 repeats.[19,39,40] In this chromosomal region, altered levels of DNA methylation have also been associated with other genetic diseases such as the Rett's syndrome and the ICF syndrome,[25] as well as in several types of cancer.[28–30]

Here, we have first studied the NM association of a 368-bp-long DNA segment containing four different SSLPs. Taken together, these SSLPs account for the majority of the genotypes identified within the FR-MAR. We found that the presence of an 8nt+ haplotypes within the polymorphic region was associated with a higher affinity for the NM. These 8nt+ haplotypes are generally not associated with the FSHD phenotype.[22–24] Next, we have analyzed the level of DNA methylation at four CpGs in the FR-MAR region in relation with NM attachment. One of the four CpGs, CpG_4, was found to be 100% methylated in the NM fraction. In total DNA, CpG_4 was also by far the most frequently methylated CpG, although with variable frequencies depending on the cells analyzed. When the cells were treated with the DNA methylation inhibitor 5-aza-dC, the affinity of the FR-MAR to the NM was reduced more than twofold, indicating that DNA methylation probably favors an efficient interaction between FR-MAR and the NM. Owing to the fact that a copy of FR-MAR is present within the q26 region of human chromosome 10,[22–24] data obtained here are valid for both 4q35 and 10q26 FR-MARs.

Chromosome looping is known to be mediated, at least in part, by MeCP2,[36,37] a methyl-cytosine-binding protein that associates with nuclear matrices.[41] MeCP2 binding sites were indeed identified within the FR-MAR. Using ChIP, we have observed that the level of MeCP2 binding to the FR-MAR was directly related with NM binding, consistent with a role of MeCP2 in FR-MAR attachment to NM. Additional elements could still have a role in NM attachment. Indeed, in the FR-MAR, the levels of histone H3 acetylated at lysine 9 were found to be inversely related with NM binding. Furthermore, when cells were treated with the histone deacetylase inhibitor TSA, the binding of the FR-MAR to the NM was significantly reduced, a direct experimental evidence of the impact of a chromatin-modifying agent on NM attachment. This effect was additive with that of 5-aza-dC, consistent with a combined role of DNA methylation and chromatin structure in NM attachment. Of note, 5-aza-dC and TSA are both known to provoke a decompaction of chromatin, which thus appears to inhibit the interaction between the FR-MAR and the NM.

In summary, three criteria seem to be important for high-affinity interaction between the FR-MAR and the NM: (i) the presence of an 8nt+ haplotype in chromosomal DNA; (ii) the methylation of one specific CpG, namely CpG_4, within the FR-MAR; and (iii) the absence of H3K9ac in the relevant chromatin fragment (Figure 4). The presence of MeCP2 molecules provides another favoring factor. Whether other proteins have an additional role in this process will require further studies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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1 Vassetzky YS, Hair A, Razin SV: Rearrangement of chromatin domains in cancer and development. *J Cell Biochem* 2000; **S35**: 54–60.
2 Razin SV, Iarovaia OV, Sjakste N *et al*: Chromatin domains and regulation of transcription. *J Mol Biol* 2007; **369**: 597–607.
3 Brun C, Dang Q, Miassod R: Studies of an 800-kilobase DNA stretch of the *Drosophila* X chromosome: comapping of a subclass of scaffold-attached regions with sequences able to replicate autonomously in *Saccharomyces cerevisiae*. *Mol Cell Biol* 1990; **10**: 5455–5463.
4 Iarovaia O, Hancock R, Lagarkova M, Miassod R, Razin SV: Mapping of genomic DNA loop organization in a 500-kilobase region of the *Drosophila* X chromosome by the topoisomerase II-mediated DNA loop excision protocol. *Mol Cell Biol* 1996; **16**: 302–308.
5 Ioudinkova E, Petrov A, Razin SV, Vassetzky YS: Mapping long-range chromatin organization within the chicken alpha-globin gene domain using oligonucleotide DNA arrays. *Genomics* 2005; **85**: 143–151.
6 Linnemann AK, Platts AE, Krawetz SA: Differential nuclear scaffold/matrix attachment marks expressed genes. *Hum Mol Genet* 2009; **18**: 645–654.
7 Drennan KJ, Linnemann AK, Platts AE, Heng HH, Armant DR, Krawetz SA: Nuclear matrix association: switching to the invasive cytotrophoblast. *Placenta* 2010; **31**: 365–372.
8 Platts AE, Quayle AK, Krawetz SA: *In silico* prediction and observations of nuclear matrix attachment. *Cell Mol Biol Lett* 2006; **11**: 191–213.
9 Mirkovitch J, Mirault ME, Laemmli UK: Organization of the higher-order chromatin loop: specific DNA attachment sites on nuclear scaffold. *Cell* 1984; **39**: 223–232.

10 Vassetzky YS, Bogdanova AN, Razin SV: Analysis of the chicken DNA fragments that contain structural sites of attachment to the nuclear matrix: DNA–matrix interactions and replication. *J Cell Biochem* 2000; **79**: 1–14.
11 Vassetzky Y, Hair A, Mechali M: Rearrangement of chromatin domains during development in *Xenopus*. *Genes Dev* 2000; **14**: 1541–1552.
12 Oberhammer F, Wilson JW, Dive C *et al*: Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. *EMBO J* 1993; **12**: 3679–3684.
13 Petrov A, Pirozhkova I, Laoudj D, Carnac G, Lipinski M, Vassetzky YS: Chromatin loop domain organization within the 4q35 locus in facioscapulohumeral dystrophy patients versus normal human myoblasts. *Proc Natl Acad Sci USA* 2006; **103**: 6982–6987.
14 Petrov AV, Allinne J, Pirozhkova IV, Laoudj D, Lipinski M, Vassetzky YS: A nuclear matrix attachment site in the 4q35 locus has an enhancer-blocking activity *in vivo*: implications for the facio-scapulo-humeral dystrophy. *Genome Res* 2008; **18**: 39–45.
15 Bode J, Maass K: Chromatin domain surrounding the human interferon-beta gene as defined by scaffold-attached regions. *Biochemistry* 1988; **27**: 4706–4711.
16 Forrester WC, Fernandez LA, Grosschedl R: Nuclear matrix attachment regions antagonize methylation-dependent repression of long-range enhancer–promoter interactions. *Genes Dev* 1999; **13**: 3003–3014.
17 Fernandez LA, Winkler M, Grosschedl R: Matrix attachment region-dependent function of the immunoglobulin mu enhancer involves histone acetylation at a distance without changes in enhancer occupancy. *Mol Cell Biol* 2001; **21**: 196–208.
18 Martens JH, Verlaan M, Kalkhoven E, Dorsman JC, Zantema A: Scaffold/matrix attachment region elements interact with a p300-scaffold attachment factor A complex and are bound by acetylated nucleosomes. *Mol Cell Biol* 2002; **22**: 2598–2606.
19 deGreef JC, Frants RR, van der Maarel SM: Epigenetic mechanisms of facioscapulohumeral muscular dystrophy. *Mutat Res* 2008; **647**: 94–102.
20 Dmitriev P, Lipinski M, Vassetzky YS: Pearls in the junk: dissecting the molecular pathogenesis of facioscapulohumeral muscular dystrophy. *Neuromusc Disord* 2009; **19**: 17–20.
21 Dmitriev P, Barat A, Cochet E *et al*: FSHD myoblasts fail to downregulate intermediate filament protein vimentin during myogenic differentiation. *Biopolym Cell* 2011; **27**: 359–363.
22 Lemmers RJ, van der Vliet PJ, Klooster R *et al*: A unifying genetic model for facioscapulohumeral muscular dystrophy. *Science* 2010; **329**: 1650–1653.
23 Lemmers RJ, Wohlgemuth M, van der Gaag KJ *et al*: Specific sequence variations within the 4q35 region are associated with facioscapulohumeral muscular dystrophy. *Am J Hum Genet* 2007; **81**: 884–894.
24 Lemmers RJ, van der Vliet PJ, van der Gaag KJ *et al*: Worldwide population analysis of the 4q and 10q subtelomeres identifies only four discrete interchromosomal sequence transfers in human evolution. *Am J Hum Genet* 2010; **86**: 364–377.
25 Forlani G, Giarda E, Ala U *et al*: The MeCP2/YY1 interaction regulates ANT1 expression at 4q35: novel hints for Rett syndrome pathogenesis. *Hum Mol Genet* 2010; **19**: 3114–3123.
26 Kondo T, Bobek MP, Kuick R *et al*: Whole-genome methylation scan in ICF syndrome: hypomethylation of non-satellite DNA repeats D4Z4 and NBL2. *Hum Mol Genet* 2000; **9**: 597–604.
27 de Greef JC, Lemmers RJ, van Engelen BG *et al*: Common epigenetic changes of D4Z4 in contraction-dependent and contraction-independent FSHD. *Hum Mutat* 2009; **30**: 1449–1459.
28 Cadieux B, Ching TT, VandenBerg SR, Costello JF: Genome-wide hypomethylation in human glioblastomas associated with specific copy number alteration, methylenetetrahydrofolate reductase allele status, and increased proliferation. *Cancer Res* 2006; **66**: 8469–8476.
29 Tsumagari K, Qi L, Jackson K *et al*: Epigenetics of a tandem DNA repeat: chromatin DNaseI sensitivity and opposite methylation changes in cancers. *Nucleic Acids Res* 2008; **36**: 2196–2207.
30 Katargin AN, Pavlova LS, Kisseljov FL, Kisseljova NP: Hypermethylation of genomic 3.3-kb repeats is frequent event in HPV-positive cervical cancer. *BMC Med Genom* 2009; **2**: 30.
31 Choi SH, Worswick S, Byun HM *et al*: Changes in DNA methylation of tandem DNA repeats are different from interspersed repeats in cancer. *Int J Cancer* 2009; **125**: 723–729.
32 Tilman G, Loriot A, Van Beneden A *et al*: Subtelomeric DNA hypomethylation is not required for telomeric sister chromatid exchanges in ALT cells. *Oncogene* 2009; **28**: 1682–1693.
33 Barro M, Carnac G, Flavier S, Mercier J, Vassetzky YS, Laoudj-Chenivesse D: Primary myoblasts derived from the facioscapulohumeral dystrophy patients are hypersensitive to oxidative stress and show defects upon terminal differentiation. *J Cell Mol Med* 2010; **14**: 275–289.
34 Gasser SM, Vassetzky YS: Analysis of nuclear scaffold attachment regions. In: Gould H (ed) *Chromatin: A Pratical Approach*. Oxford, UK: Oxford University Press, 1998; pp 111–124.
35 Stratling WH, Yu F: Origin and roles of nuclear matrix proteins. Specific functions of the MAR-binding protein MeCP2/ARBP. *Crit Rev Eukaryot Gene Expr* 1999; **9**: 311–318.
36 Horike S, Cai S, Miyano M, Cheng JF, Kohwi-Shigematsu T: Loss of silent-chromatin looping and impaired imprinting of DLX5 in Rett syndrome. *Nat Genet* 2005; **37**: 31–40.
37 Eivazova ER, Gavrilov A, Pirozhkova I *et al*: Interaction *in vivo* between the two matrix attachment regions flanking a single chromatin loop. *J Mol Biol* 2009; **386**: 929–937.
38 Klose RJ, Sarraf SA, Schmiedeberg L, McDermott SM, Stancheva I, Bird AP: DNA binding selectivity of MeCP2 due to a requirement for A/T sequences adjacent to methyl-CpG. *Mol Cell* 2005; **19**: 667–678.
39 Zeng W, de Greef JC, Chen YY *et al*: Specific loss of histone H3 lysine 9 trimethylation and HP1gamma/cohesin binding at D4Z4 repeats is associated with facioscapulohumeral dystrophy (FSHD). *PLoS Genet* 2009; **5**: e1000559.
40 van Overveld PG, Lemmers RJ, Sandkuijl LA *et al*: Hypomethylation of D4Z4 in 4q-linked and non-4q-linked facioscapulohumeral muscular dystrophy. *Nat Genet* 2003; **35**: 315–317.
41 Weitzel JM, Buhrmester H, Stratling WH: Chicken MAR-binding protein ARBP is homologous to rat methyl-CpG-binding protein MeCP2. *Mol Cell Biol* 1997; **17**: 5656–5666.

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REVIEW

Histone deacetylase inhibitors and epigenetic regulation in lymphoid malignancies

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Abstract A vast majority of lymphomas and leukaemias are results of translocations. These translocations produce various genetic and epigenetic changes that lead to oncogenesis. This opens an opportunity to use a relatively new class of anti-cancer agents, inhibitors of histone deacetylases (HDACi) to target lymphoid malignancies. Surprisingly, the rational basis for treatment of lymphomas with HDACi is far from clear, although some positive results have been obtained. Here we analyze the effect of histone deacetylase (HDAC) inhibitors on lymphoid malignancies.

Keywords Epigenetics · Histone deacetylase inhibitors · Lymphoma

Introduction

Gene regulation is accomplished by a combination of genetically determined elements (promoters, enhancers, silencers, locus control elements etc.) and factors that are not directly encoded in DNA, such as DNA methylation and histone modification. The patterns of DNA methylation and chromatin might be transitory, but might also be transmitted through one or several generations or cell divisions. In this case, these changes are called epigenetic, the definition of epigenetics was proposed by Conrad Waddington in 1942 [1]. Recent studies established a link between epigenetics and cancer. Indeed, changes in the pattern of DNA methylation or histone acetylation often precede or accompany malignant transformation [2].

Lymphomas and leukaemias represent an interesting class of cancers where almost 100 % of malignant transformation is due to chromosomal translocations (for review see [3]). Chromosomal translocations may result in three possible scenarios. First, deregulation of important genes, and particularly proto-oncogenes and tumor suppressor genes crucial for regulation of important cellular processes [4, 5] which may occur either by juxtaposition of oncogenes to a transcription control element of another gene on a different chromosome, thereby leading to an abnormal expression of the translocated gene [3], or by relocalization of the translocated region in the nuclear space [6, 7]. Second, the translocation may also results in the formation of an unique fusion gene, which in turn codes an activated form of the protein that affects the normal cellular physiology [8–10]. Third, some translocations, particularly non-reciprocal ones, may lead to changes in the gene dosage, i.e., a loss of tumour suppressor genes or duplication of oncogenes [11]. Some translocations could also lead to pointed or large-scale epigenetic changes. Below we shall consider several known levels of epigenetic regulation, their mechanisms and link to lymphoid malignancies and lymphomagenesis.

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DNA methylation

In higher eukaryotes, the cytosine within the dinucleotide CpG can be methylated. CpG dinucleotides are often clustered in the genome into so-called CpG islands ranging from 0.5 to 5 kb in size. CpG islands often contain control elements for transcription [12] and replication [13]. Usually transcription is silenced when CpG islands are methylated. The constant level of DNA methylation is maintained by DNA methyltransferase 1 (DNMT1) and *de novo* DNA methylation is catalyzed by DNMT 3A and 3B. Extensive studies of DNA methylation have revealed hundreds of individual genes aberrantly methylated in different lymphomas and leukaemias (reviewed in [14, 15]). DNA methylation maintains as well repetitive sequences of the genome in stable state. If this mechanism is altered it leads to genomic instability and hence to development of different malignancies [16].

Histone modifications

Genomic DNA is wrapped around a protein octamer forming a nucleosome. Nucleosomes consist of ~150 base pairs of genomic DNA and core histones H2A, H2B, H3, and H4. The bodies of the histones are inside the nucleosome, but their tails protrude. These tails may undergo many posttranslational chemical modifications. A combination of these modifications such as acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation produces important signaling information called "histone code." It can alter the chromatin structure and affect many biological processes [17].

Histone methylation

Reversible histone methylation is catalyzed by S-adenosylmethionine (SAM) - dependent methyltransferases and erased by either the Jumonji family of demethylases or the lysine-specific histone demethylases 1 (LSD1) and 2 (LSD2) ([18, 19]). Lysine-(K) and arginine-(R) residues can be methylated. Histone methylation ensures binding of regulatory proteins that affect chromatin structure in different ways. Trimethylation of H3 histone at lysine 4 is linked to transcription, at the same time trimethylation of H3 at lysine 27 is associated with gene silencing [17].

A lot of evidence link histone methylation with lymphoid malignancies: several genes amplified in these cancers are histone methyltransferases. MLL, a H3 K4 methyltransferase, is frequently mutated or duplicated in acute leukaemias [20]. EZH2, a component of the Polycomb repressive complex (PRC) is a H3K27 methyltransferase frequently over-expressed in several types of leukemia [21] and B-cell lymphoma [22]. EZH2 was also reported to be mutated in some myeloid malignancies [23, 24].

Histone acetylation

Histone acetylation is also a reversible process catalyzed by histone acetyltransferases (HAT) and histone deacetylases (HDAC). HATs use acetyl-CoA as a cofactor to acetylate either arginine-(R) or lysine-(K) residues. Acetylated chromatin has a relaxed structure that is favorable for transcription and may interact with bromodomain-containing family of proteins that in turn can act as transcription activators or chromatin remodelers [25]. Altered activity of HATs and HDACs lead to disturbed balance in chromatin modifications what can affect the gene expression of pro-apoptotic genes and proto-oncogenes which in its turn contributes to the lymphomagenesis. Indeed the histone acetylation level is perturbed in different types of cancer [26].

Decreased HAT activity caused by gene deletions and somatic mutations together with gain-of-function mutations of HMTs leads to repressed chromatin states linked to malignant processes in lymphoid malignancies [27]. Two highly related HATs, CREBBP and EP300 often harbor mutations in their HAT coding domain in follicular and diffuse large B-cell lymphoma. The same study pointed the importance of allelic dosage of HAT genes [28]. There are several examples of translocations involving HATs such as MLL-CBP and MOZ-TIF2; altered expression of HAT genes is observed in a range of cancers (reviewed in [29]).

Histone deacetylases

The removal of acetyl groups from arginine and lysine residues in histones are catalyzed by HDACs. This group comprising in humans 18 enzymes is quite heterogeneous and can be divided into four classes based on their sequence homology to their yeast orthologues: Rpd3, yHda1 and Sir2, respectively (Table 1).

The Class I, II, and IV HDACs, called as well «classical» HDACs, belong to the arginase/deacetylase superfamily of proteins which require Zn2+ for their catalytic activity. Class I HDACs are nucleus located, the most abundant and ubiquitously-expressed. Class II HDAC shuttle between the nucleus and the cytoplasm and their expression is tissue-specific. That class can be divided on two sub-classes: IIa is characterized by having a highly conserved C-terminal deacetylase catalytic domain and unique N-terminal domain; IIb contains two deacetylase domains. Class IV has only one member HDAC11 and resides in the nucleus [31]. Class III, or sirtuins, belongs to deoxyhypusine synthase like NAD/FAD-binding domain superfamily and uses NAD+ as an essential co-factor.

Table 1 Human HDACs and their functions

HDAC	Protein associations (normal and oncogenic)	Role in the cell	Expression in tumor tissues	Function in cancer cells	References
Class I (homologous to RDP3 yeast protein, nuclear location, ubiquitous tissue expression, co-factor for activity - Zn2+)					
HDAC1 (482 a.a.)	HDAC2, CoREST, NuRD, Sin3, AML1-ETO, PML, PLZF, BCL6, p53, AR, ER, Rb/E2F1, MyoD, STAT3, androgen	Represses transcription, binds to transcription factors. Resistance to chemotherapy Proliferation control Apoptosis p21 and p27 CDK inhibitor repression	Especially common in cancers of the gastrointestinal system and is associated with dedifferentiation, enhanced proliferation, invasion, advanced disease and poor prognosis. DLBCL, ALL: high expression is associated with poor prognosis.	KD results in inhibition of proliferation and induction of autophagy. KD results in abrogated lymphomagenesis due to a block in early thymic development.	[30] [31] [32] [33] [34] [35]
HDAC2 (488 a.a.)	HDAC1, CoREST, NuRD, Sin3, AML1-ETO, PML, PLZF, Bcl6, STAT3, glucocorticoid receptor, YY-1	Negatively regulates transcription by being recruited to DNA as a corepressor Proliferation control Apoptosis	Gastric, prostate, colorectal cancers, DLBCL, ALL: high expression is associated with poor prognosis.	Cervical cancer cells: HDAC2 KD results in differentiation, apoptosis and p53 independent p21 expression. Breast cancer cells: increased p53 activity, inhibition of proliferation, induction of senescence, induction of apoptosis. Colon cancer cells: KD causes growth arrest. Neuroblastoma cells: KD induces apoptosis. Genetic HDAC2 mutation reduces intestinal tumor development in APC mice in vivo. KD results in abrogated lymphomagenesis due to a block in early thymic development. CLL cells: KD sensitizes for TRAIL-apoptosis	[30] [31] [32] [33] [36]
HDAC3 (428 a.a.)	HDAC4, HDAC5, HDAC7, NCoR/SMRT, AML1-ETO, PML, PLZF, PML-RARα, PLZF-RARα, Bcl6, STAT1, TAT3, GATA1, GATA2, NF-κB, RelA, MEF2D, YY-1, SHP	Represses transcription, binds to transcription factors Proliferation Differentiation	Gastric, prostate, colorectal cancers: high expression associated with poor prognosis.	APL cells: HDAC3 associated with PML-RARα fusion protein, KD induces differentiation genes. AML: AML-1-ETO binds HDAC3 (and HDACs 1, 2), disrupts cell cycle.	[30] [32] [31] [33]
HDAC8 (377 a.a.)	nd	Proliferation Differentiation	Childhood neuroblastoma: high HDAC8 expression significantly correlates with advanced stage disease, clinical and genetic risk factors and poor long term survival.	Neuroblastoma cells: HDAC8 KD induces differentiation, cell cycle arrest and inhibits clonogenic growth. Lung, colon, cervical cancer cells: KD of HDAC8 reduces proliferation. HDAC8 specific inhibitor selectively induces apoptosis in T-cell derived lymphoma and leukemic cells.	[30] [31] [32] [33]
Class IIa (homologous to Hda1 yeast protein, shuttle between nucleus and cytoplasm, tissue-restricted expression, co-factor for activity - Zn2+)					
HDAC4 (1084 a.a.)	HDAC3-NCoR, GATA1, GCMa, HP-1	Differentiation Angiogenesis	ALL: high expression is associated with poor prognosis, high initial leukocyte count, T-cell ALL and prednisone poor-response.	APL cells: HDAC4 interacts with PLZF-RARα fusion protein, represses differentiation genes. Renal carcinoma cells: KD inhibits expression and functional activity of HIF-1a.	[30] [31] [32] [33] [37]
HDAC5 (1122 a.a.)	HDAC3-NCoR, GATA1, GATA2, Smad7, HP-1, GCMa	Differentiation	Up-regulated in colorectal cancer. Down-regulated in AML and lung cancer.	Erythroleukemia: HDAC5 shuttles from nucleus to cytoplasm upon differentiation, interacts with GATA-1. KD decreased medullablastoma cell growth and viability.	[30] [31] [32] [33]
HDAC7	HDAC3-NCoR, ERα, FLAG1 and 2	Angiogenesis			[30]

Table 1 (continued)

HDAC	Protein associations (normal and oncogenic)	Role in the cell	Expression in tumor tissues	Function in cancer cells	References
(855 a.a.)			Up-regulated in colorectal cancer and ALL. Down-regulated in lung cancer.	Endothelial cells: HDAC7 silencing alters morphology, migration and tube-forming capacity. KD induced growth arrest in colon and breast cancer cells.	[31] [32] [33]
HDAC9 (1011 a.a.)	nd		Over-expressed in ALL and medulloblastoma, associated with poor prognosis.	KD of HDAC9/10 inhibited homologous recombination and increased sensitivity to DNA damage and decreased medullablastoma cell growth and viability	[30] [31] [32] [33]
Class IIb (homologous to yeast protein Hda1, mostly cytoplasmic location, tissue-restricted expression, co-factor for activity - Zn2+)					
HDAC6 (1215 a.a.)	HDAC11, α-Tubulin, HSP90, SHP, Smad7	Regulation of protein degradation both via the aggresome (a structure that forms in response to misfolded proteins) and the regulation of Hsp90 chaperone activity. Angiogenesis. Migration.	Oral squamous cell cancer: high expression, increased in advanced stage. Up-regulated in breast cancer and CTCL. In breast cancer is associated with enhanced prognosis. Down-regulated in lung cancer.	K562 leukemic cells: targeted inhibition of HDAC6 leads to acetylation of HSP90 and disruption of its chaperone function, resulting in depletion of pro-growth and pro-survival client proteins including the Bcr-Abl oncoprotein. Colon carcinoma cells: HDAC6 targeting blocks EGF induced nuclear translocation of ß-catenin and c-myc expression. KD of HDAC6 causes donwregulation of HIF-1a, VEGFR1/2. HDAC6 involved in TGFβ induced epithelial-mesenchymal transition of lung carcinoma cells.	[30] [31] [32] [33]
HDAC10 (669 a.a.)	HSP90	Angiogenesis	Overexpressed in hepatocellular carcinoma. Poor prognostic indicator in lung cancer.	KD of HDAC10 downregulates VEGFR.	[38] [31] [32] [33]
Class III or sirtuins (Silent information regulator 2 family gemology, co-factor for activity - NAD+, conventional HDACis do not affect them)					
SIRT1 (747 a.a.) (Nucleus)	H3K9 H3K14 H3K56 H4K16 H1K26 p53 KU70 Foxo3a BCL6	Chromatin organization, DNA repair/genome stability, stress, cancer	Upregualted in human lung cancer, prostate cancer and leukemia and has been found downregulated in colon tumors.		[39] [40] [41]
SIRT2 (352 a.a.) (Cytoplasm)	H4K16 H3K56	Chromatin condensation, mitosis, DNA repair, cancer	Frequently downregulated in human gliomas.		[39] [41]
SIRT3 (399 a.a.) (Nucleus and mitochondria)	H4K16	Chromatin silencing, DNA repair, cellular stress			[39]
SIRT4 (310 a.a.) (Mitochondria)	None				[39]

Table 1 (continued)

HDAC	Protein associations (normal and oncogenic)	Role in the cell	Expression in tumor tissues	Function in cancer cells	References
SIRT5 (314 a.a.) (Mitochondria)	None				[39]
SIRT6 (355 a.a.) (Nucleus)	H3K9 H3K56	Telomeric chromatin/senescence, DNA repair/genome stability			[39]
SIRT7 (400 a.a.) (Nucleus)	H3K18	Cellular transformation			[39]
Class IV (Unknown yeast protein homology, cytoplasmic location, tissue-restricted expression, co-factor for activity - Zn2+)					
HDAC11 (347 a.a.)	HDAC6	Regulates the protein stability of DNA replication factor CDT1 and the expression of interleukin-10.	Over-expressed in breast, renal and liver cancer. ALL: high expression is associated with poor prognosis.	It has been implicated in immune system regulation via its role in interleukin-10 expression and OX40L surface expression in Hodgkin lymphoma	[30] [38] [31] [32] [42] [33]

KD knockdown, *ALL* acute lymphoblastic leukemia, *AML* acute myeloid leukemia, *APL* acute promyelocytic leukemia, *CLL* chronic lymphocytic leukemia, *DLBCL* diffuse large B-cell lymphoma

Apart of histone deacetylation, sirtuins have mono-ADP-ribosyltransferase enzymatic activity [39].

While purified to homogeneity, classical HDACs reveal very low histone deacetylase activity which makes difficult to define their histone substrate specificity. HDACs exist as a part of several different complexes, where each of them shows different substrate preferences. In contrast to the classical ones, class III HDACs have a clearer histone substrate specificity (Table 1).

All types of HDACs, apart from regulating histone modification, also regulate a wide range of non-histone proteins working in nucleus, cytoplasm and mitochondria: such as chaperones, transcription factors and structural proteins [39, 43]. For example, acetylation of Lys residue is competitive for other its modifications, thus it inhibits ubiquitination dependent protein degradation [44]. A large number of proteins sensitive for the regulation by acetylation have been recently identified [31, 45]. Such key cell survival proteins as p53, Ku70, BCL6, tubulin turn to be targets for classical HDACs and sirtuins [46–48]. Therefore, altered HDAC activity may influence both gene expression and other cellular processes including tumor cell apoptosis, growth arrest, differentiation, inhibition of angiogenesis, etc. [33].

Abnormal HDACs play a key role in many human diseases. Class I and Class II HDACs were implicated in several types of cancer, including lymphomas (reviewed in [43]).

Activity of HDACs is very often altered in lymphoid malignancies whether by overexpression, mutations or by involving in the complex with fusion oncoproteins. Chimeric fusion proteins, such as PML-RARa, PLZF-RAR , and AML1-ETO, the result of chromosomal translocations in some leukemia cases, usually recruit HDACs for their activity leading to gene silencing [29, 49, 50]. The expression of different HDACs itself has been observed to be up-regulated in various types of cancer [31, 33]. For example, chronic lymphocytic leukemia is characterized with generally increased HDAC expression [51]. Although mutations in HDACs are relatively rare, mutations in HDAC2 and HDAC4 were reported in human epithelial cancer cell lines and breast cancer, respectively [38].

Histone deacetylase inhibitors and lymphomas

One of the reasons which makes the treatment with HDAC inhibitors favorable is that normal cells are relatively resistant to the treatment, whereas cancer cells are more responsive and undergo growth arrest, inhibited differentiation and apoptosis.

The mechanisms of action of HDAC inhibitors till date are not all completely discovered. The most studied for now concern selective alterations of gene expression and post-translational regulation of pro- and anti-apoptotic genes from «extrinsic» (death-receptor) and «intrinsic» (mitochondrial)

pathways of apoptosis, inhibition of angiogenesis, generation of reactive oxygen species (ROS), autophagy and other [31, 52].

The first inhibitor of HDACs, n-Butyrate was described in the seventies before the actual discovery of histone deacetylases [53]. Several other drugs targeting histone acetylation, including Trychostatin A (TSA) and valporoic acid (VPA) were discovered and introduced into clinical practice for treatment of diseases non-connected with cancer. Discovery of the link between histone acetylation and cancer has sparked an interest for HDACi and several new drugs were developed during last 10 years. These drugs can be divided into several chemically distinct groups: aliphatic acids (phenylbutyrate, valporoic acid), benzamides (entinostat), cyclic peptides (romidepsin), and hydroxamates (TSA, vorinostat/SAHA). The HDAC inhibitors that have been tested in lymphoid malignancies are summarized in Table 2.

Vorinostat or SAHA is the first HDACi approved by the United States Food and Drug Administration (U.S. FDA) in 2006 for clinical use in patients with cutaneous T-cell lymphoma (Zolinza®, Merck and Co., Inc.) [54, 55]. First synthesized in 1998 it was shown to induce erythroid differentiation [56]. SAHA is pan-HDAC inhibitor that blocks all Class I, II, and IV enzymes. It was also tested in Hodgkin lymphoma, follicular lymphoma, marginal zone lymphoma and mantle cell lymphoma with promising results relapsed/refractory indolent follicular lymphoma [57, 58]. Vorinostat belongs to the hydroxamic acid group.

Romidepsin (FK228) is a cyclic peptide isolated from *Chromobacterium violaceum* ([59]). It has been investigated in patients with hematological malignancies such as CML, AML, MDS, multiple myeloma, cutaneous T-cell lymphoma (CTCL) and peripheral T-cell lymphoma (PTCL) [60, 61]. The latter has showed promising results [62]. In 2009, it has been granted U.S. FDA approval for CTCL treatment, and for PTCL in 2011 (Istodax®, Gloucester Pharmaceuticals—a subsidiary of Celgene Corp). A good correlation between drug exposure level and histone acetylation has been revealed [63].

Belinostat (PXD-101), another hydroxamic acid derivative HDAC inhibitor, has shown clinical response in patients with B-cell lymphoma [64], mantle cell lymphoma and HL [65], PTCL and CTCL [66]. It is the third drug received in 2014 the U.S. FDA approval for the treatment of refractory or relapsed PTCL (Beleodaq®, Spectrum Pharms, Inc.) [67].

Chidamide (CS055) is a new benzamide type of HDAC inhibitor with subtype selective activity against HDAC1, 2, 3 and 10. Chidamide is being studied in multiple clinical trials as a single agent or in combination with chemotherapeutic agents for the treatment of various hematological and solid cancers [68, 69]. It induces of growth arrest and apoptosis in blood and lymphoid-derived tumor cells, activates NK- and CD8 T-cell-mediated antitumor activity, stimulates differentiation of tumor stem cells and represses genes associated with drug resistance [68]. In 2015 chidamide obtained approval (Epidaza®) from the Chinese FDA for the treatment of relapsed or refractory PTCL.

Givinostat is a hydroxamic pan HDAC inhibitor recently tested in several hematological malignancies such as multiple myeloma, HL and myeloproliferative disease. Clinical efficacy has been reported in patients with Polycythemia Vera and Essential Thrombocythemia ([70]).

Panobinostat is also a hydroxamic acid analog with pan HDAC inhibitor activity. It has been studied in various hematological malignancies with revealing good clinical results in patients with CTCL [71], HL [72], AML [73]. Panobinostat was shown to induce autophagy, thus combination of that drug with autophagy inhibitor can increase its antitumour effects [74].

Valproic Acid (VPA) is a member of the short chain fatty acids, inhibitor of HDACs class I. VPA is a potent and very selective inhibitor of STAT3 [75]. It showed cell-killing activity by triggering apoptotic pathways in preclinical studies on chronic lymphocytic leukaemia (CLL) [76]. VPA is undergoing evaluation in India as therapy for CLL.

Entinostat, a benzamide derivative class member specific to class I HDACs, has been tested in several lymphomas [77]. It is tolerated well both alone and in combination with other drugs [78].

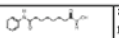
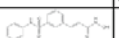
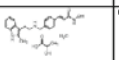
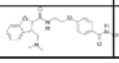
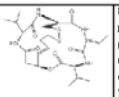
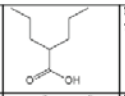
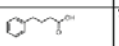
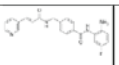
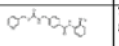
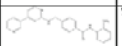
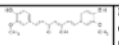
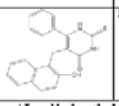
Mocetinostat is another synthetic benzamide with an inhibiting specificity for HDACs class I and IV. It has a prolonged pharmacodynamic effect [79]. This drug was evaluated for the clinical efficacy in patients with follicular lymphoma, Hodgkin lymphoma [80] and AML [81].

Abexinostat, or S78454, is a novel hydroxamic acid pan HDAC inhibitor. Abexinostat induces apoptosis and histone alterations in a caspase-8 depended manner in acute leukemia cells [82]. In clinical studies Abexinostat showed promising durable responses in follicular lymphoma, B-cell lymphoma, chronic lymphocytic leukaemia patients and is currently undergoing phase II of clinical trials [83].

Cambinol, a sirtuin inhibitor, has been reported to have antitumor activity in Burkitt's lymphoma by increasing p53 acetylation [40].

The first generation of HDACis blocks most part of HDACs from class I, some of which block class II as well, while still fewer also block class IV. Such HDACis are called pan-inhibitors, because of their property of inhibiting several classes. In solid tumors, it is often reported that altered expression of individual HDACs takes place. For example, in gastric cancer HDAC1 and 2 are over-expressed, in breast cancer up-regulated HDACs 6 and 8 are associated with increased invasion [52]. These discoveries provoked increased interest for synthesis of class-selective or isoform-selective inhibitors. Some examples of such selective inhibitors are: tubacin, which increases acetylation of tubulin, but

Table 2 HDAC inhibitors

Drug	HDAC Target (Potency)	Type of cancer in which tested	Clinical trial phase	Structure	Mechanism of action	References
Hydroxamic Acids						
Vorinostat (SAHA)	Class I, II (μM)	Hodgkin's lymphoma, non-Hodgkin's lymphoma and multiple myeloma	Food and Drug Administration approved for CTCL I-II-III		Suppresses genes that promote uncontrolled growth, promotes apoptosis, interrupts cell cycle progression. TD; GA; AI; AE; MF; AU; S; PP; ROS-CD	[97] [98]
Belinostat (PXD-101)	Class I, II	B-cell lymphoma, MCL, HL, PTCL and CTCL	FDA approval for Refractory or Relapsed Peripheral T cell lymphoma.		GA; A	[55] [58] [57] [56]
Panobinostat (LBH-589)	Class I and II (nM)	CTCL, PTCL, myelogenous leukaemia	I-II-III		GA; A; ROS-CD	[97] [98]
Abexinostat (PCI-24781, CRA-024781)	Class I and II	B-cell lymphoma	II			[75] [98] [74]
Cyclic Peptides						
Romidepsin (FK228)	Class I and II (nM)	CML, AML, MDS, multiple myeloma, CTCL and PTCL	FDA approval for CTCL treatment		Suppresses genes that promote uncontrolled growth, promotes apoptosis, interrupts cell cycle progression. TD; GA; A; AI; AE; MF; ROS-CD	([50] [51] [53] [52] [54] [99] [97]
Aliphatic Acids						
Valproic Acid	Class I and IIa (mM)	Myelodysplastic syndromes Lymphocytic leukaemia	I-II		Induces oxidative stress. TD; GA; A; S	[97] [100] [98]
Pivanex (AN-9)	μM	T-ALL Chronic myelocytic leukemia	I - II		TD; GA; A	[97] [98]
						[101]
Sodium phenyl butyrate (NaPB)	Class I and IIa (mM)	Myelodysplastic syndromes	I		TD; GA; A; AI; AE	[97] [102] [98]
Benzamides						
Chidamide (CS055)	Class I and IIb (nM)	PTCL	Chinese FDA approval for peripheral T-cell lymphoma			[59]
Entinostat (MS-275)	Class I (μM)	Lymphoid malignancies	I-II		TD; GA; A; AI; AE; ROS-CD	[97] [98] [102]
Mocetinostat (MGCD0103)	Class I, IV (μM)	Hodgkin's disease, advanced leukemia	II		TD; GA; A	[100] [97] [98] [103]
Tenovin-6	SIRT1 and	CML, ALL	preclinical		Induces p53 acetylation, inhibits tumor growth,	[80] [31]
	SIRT2				and eliminates cancer stem cells in combination with imatinib	[104]
Other						
Curcumin	Class I	Hodgkin's lymphoma, non-Hodgkin's lymphoma	preclinical		Induces apoptosis in cancer cells, has anti-inflammatory and anti-oxidant properties	[105] [98]
Cambinol	SIRT1 and SIRT2	BL	preclinical		Increases p53 acetylation	[75] [31]

Abbreviations: *GA* growth arrest, *TD* terminal differentiation, *A* apoptosis, *AI* cell death by activating intrinsic apoptotic pathway, *AE* cell death by activating extrinsic apoptotic pathway, *MF* mitotic failure, *AU* autophagic cell death, *S* senescence, *PP* polyploidy, *ROS-CD* reactive oxygen species – facilitated cell death, *CTCL* of cutaneous T-cell lymphoma, *PTCL* peripheral T-cell lymphoma, *BL* Burkitt lymphoma, *CML* chronic myelogenous leukemia, *MDS* Myelodysplastic syndrome, *MCL* mantle cell lymphoma, *ALL* acute lymphoblastic leukemia

not that of histones [84]; PCI-34051 which is specific to HDAC8 and is shown to induce apoptosis in T-cell lymphoma without affecting histone or α-tubulin acetylation [85]; and benzamide inhibitor SHI-1:2 which is selective to HDAC1/HDAC2 [86]. But complete understanding and characterization of HDAC expression patterns in various cancers is still far from being reached. Moreover, in hematological malignancies HDAC expression patterns are not clearly distinguishable [51]. All that makes it difficult to intelligently target specific HDACs up to date.

The effect of HDACi on lymphoid malignancies can be improved by combination with other drugs, e.g., DNA damaging agents such as topoisomerase II poisons or DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (Decitabine) [87]. Tenovin-6, sirtuin inhibitor, together with BCR-ABL tyrosine kinase inhibitor (Imatinib) was shown to be effective in chronic myelogenous leukemia [88]. A combination of new potent HDAC inhibitors, including abexinostat, belinostat, panobinostat, entinostat and vorinostat with 5-aza-2′-deoxycytidine may lead to an increase in clinical benefits.

HDACi and epigenetic regulation in lymphoid malignancies

Lymphoid malignancies are provoked by translocations (for review see [3]). Some of these translocations result in gene fusions. Most gene fusions involve transcription factors or chromatin regulators. In myeloid leukemias, the fusion proteins such as PML-RAR and AML1-ETO form stable complexes with HDACs [8–10]. This provided a rational basis for the first study of HDACi in leukemia. Treatment of mice with PML-RAR induced leukemia with valporoic acid increased survival and induced apoptosis of leukemic cells [89]. This study has prompted other researchers to test HDACi on other lymphomas and leukaemias, although the mechanisms of these lymphomas are different from myeloid leukaemias. Indeed, in B-cell lymphomas, juxtaposition of gene regions may lead to over-expression of oncogenes such as *CCND1*, *CMYC* [90, 91] or *BCL2* [92, 93]. Recently, it has been shown that translocations also lead to large-scale epigenetic changes [94], large-scale reorganization of nuclear organization and aberrant gene expression [6, 7]. At the same time, most documented effects of HDACi relate to apoptosis associated with increased expression of pro-apoptotic genes and decreased expression of anti-apoptotic genes, leading to cell death via one of the following pathways: inhibition of angiogenesis, generation of reactive oxygen species, autophagy or apoptosis. Currently our understanding of the immediate and prolonged action of HDACi on cells is insufficient and further studies in this direction are required to make full advantage of these powerful anticancer drugs.

Perspectives and conclusions

HDACi represent an interesting group of anticancer drugs. Their use, alone or in combinations has a high potential in treatment of lymphoid malignancies. Development of new may improve the outcome of treatment. The action of HDACi is not limited to histones; they also deacetylate other nonhistone proteins [95]. This could both produce an additional window of opportunity for the use of HDACi, since they also target transcription factors, signal transducers, or oncoproteins including p53, YY1, and STAT3 [96], but this may also induce toxicity or counteract the effect of increased histone acetylation induced by these drugs. The epigenetic effect of HDACi on local and global chromatin organization in translocation regions in normal and lymphoid cells is insufficiently studied and is needed to better understand the mechanism of action of HDACi and develop

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Conflict of interest Authors declare no conflict of interest.

References

1. Waddington C (1942) The epigenotype. Endeavour 1:18–20
2. Esteller M (2011) Epigenetic changes in cancer. F1000 Biol Rep 3:9. doi:10.3410/B3-9
3. Nussenzweig A, Nussenzweig MC (2010) Origin of chromosomal translocations in lymphoid cancer. Cell 141(1):27–38. doi:10.1016/j.cell.2010.03.016
4. Korsmeyer SJ (1992) Chromosomal translocations in lymphoid malignancies reveal novel proto-oncogenes. Annu Rev Immunol 10:785–807. doi:10.1146/annurev.iy.10.040192.004033
5. Mitelman F, Johansson B, Mertens F (2004) Fusion genes and rearranged genes as a linear function of chromosome aberrations in cancer. Nat Genet 36(4):331–334. doi:10.1038/ng1335
6. Harewood L, Schutz F, Boyle S, Perry P, Delorenzi M, Bickmore WA, Reymond A (2010) The effect of translocation-induced nuclear reorganization on gene expression. Genome Res 20(5):554–564. doi:10.1101/gr.103622.109
7. Allinne J, Pichugin A, Iarovaia O, Klibi M, Barat A, Zlotek-Zlotkiewicz E, Markozashvili D, Petrova N, Camara-Clayette V, Ioudinkova E, Wiels J, Razin SV, Ribrag V, Lipinski M, Vassetzky YS (2014) Perinucleolar relocalization and nucleolin as crucial events in the transcriptional activation of key genes in mantle cell lymphoma. Blood 123(13):2044–2053. doi:10.1182/blood-2013-06-510511
8. Grignani F, De Matteis S, Nervi C, Tomassoni L, Gelmetti V, Cioce M, Fanelli M, Ruthardt M, Ferrara FF, Zamir I, Seiser C, Grignani F, Lazar MA, Minucci S, Pelicci PG (1998) Fusion proteins of the retinoic acid receptor-alpha recruit histone deacetylase in promyelocytic leukaemia. Nature 391(6669):815–818. doi:10.1038/35901
9. Minucci S, Maccarana M, Cioce M, De Luca P, Gelmetti V, Segalla S, Di Croce L, Giavara S, Matteucci C, Gobbi A, Bianchini A, Colombo E, Schiavoni I, Badaracco G, Hu X, Lazar MA, Landsberger N, Nervi C, Pelicci PG (2000)

Oligomerization of RAR and AML1 transcription factors as a novel mechanism of oncogenic activation. Mol Cell 5(5):811–820
10. Lin RJ, Evans RM (2000) Acquisition of oncogenic potential by RAR chimeras in acute promyelocytic leukemia through formation of homodimers. Mol Cell 5(5):821–830
11. Artandi SE, Chang S, Lee SL, Alson S, Gottlieb GJ, Chin L, DePinho RA (2000) Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. Nature 406(6796):641–645. doi:10.1038/35020592
12. Bird A (2002) DNA methylation patterns and epigenetic memory. Genes Dev 16(1):6–21
13. Prioleau MN (2009) CpG islands: starting blocks for replication and transcription. PLoS Genet 5(4), e1000454. doi:10.1371/journal.pgen.1000454
14. Taylor KH, Briley A, Wang Z, Cheng J, Shi H, Caldwell CW (2013) Aberrant epigenetic gene regulation in lymphoid malignancies. Semin Hematol 50(1):38–47. doi:10.1053/j.seminhematol.2013.01.003
15. Hatzimichael E, Crook T (2013) Cancer epigenetics: new therapies and new challenges. J Drug Deliv 2013:9. doi:10.1155/2013/529312
16. Goelz SE, Vogelstein B, Hamilton SR, Feinberg AP (1985) Hypomethylation of DNA from benign and malignant human colon neoplasms. Science 228(4696):187–190
17. Kouzarides T (2007) Chromatin modifications and their function. Cell 128(4):693–705. doi:10.1016/j.cell.2007.02.005
18. Tsukada Y, Fang J, Erdjument-Bromage H, Warren ME, Borchers CH, Tempst P, Zhang Y (2006) Histone demethylation by a family of JmjC domain-containing proteins. Nature 439(7078):811–816. doi:10.1038/nature04433
19. Shi Y, Lan F, Matson C, Mulligan P, Whetstine JR, Cole PA, Casero RA, Shi Y (2004) Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell 119(7):941–953. doi:10.1016/j.cell.2004.12.012
20. Rege-Cambrin G, Giugliano E, Michaux L, Stul M, Scaravaglio P, Serra A, Saglio G, Hagemeijer A (2005) Trisomy 11 in myeloid malignancies is associated with internal tandem duplication of both MLL and FLT3 genes. Haematologica 90(2):262–264
21. Simon JA, Lange CA (2008) Roles of the EZH2 histone methyltransferase in cancer epigenetics. Mutat Res 647(1–2):21–29. doi:10.1016/j.mrfmmm.2008.07.010
22. Sneeringer CJ, Scott MP, Kuntz KW, Knutson SK, Pollock RM, Richon VM, Copeland RA (2010) Coordinated activities of wild-type plus mutant EZH2 drive tumor-associated hypertrimethylation of lysine 27 on histone H3 (H3K27) in human B-cell lymphomas. Proc Natl Acad Sci U S A 107(49):20980–20985. doi:10.1073/pnas.1012525107
23. Ernst T, Chase AJ, Score J, Hidalgo-Curtis CE, Bryant C, Jones AV, Waghorn K, Zoi K, Ross FM, Reiter A, Hochhaus A, Drexler HG, Duncombe A, Cervantes F, Oscier D, Boultwood J, Grand FH, Cross NC (2010) Inactivating mutations of the histone methyltransferase gene EZH2 in myeloid disorders. Nat Genet 42(8):722–726. doi:10.1038/ng.621
24. Nikoloski G, Langemeijer SM, Kuiper RP, Knops R, Massop M, Tonnissen ER, van der Heijden A, Scheele TN, Vandenberghe P, de Witte T, van der Reijden BA, Jansen JH (2010) Somatic mutations of the histone methyltransferase gene EZH2 in myelodysplastic syndromes. Nat Genet 42(8):665–667. doi:10.1038/ng.620
25. Filippakopoulos P, Picaud S, Mangos M, Keates T, Lambert JP, Barsyte-Lovejoy D, Felletar I, Volkmer R, Muller S, Pawson T, Gingras AC, Arrowsmith CH, Knapp S (2012) Histone recognition and large-scale structural analysis of the human bromodomain family. Cell 149(1):214–231. doi:10.1016/j.cell.2012.02.013
26. Seligson DB, Horvath S, Shi T, Yu H, Tze S, Grunstein M, Kurdistani SK (2005) Global histone modification patterns predict risk of prostate cancer recurrence. Nature 435(7046):1262–1266. doi:10.1038/nature03672
27. Hassler MR, Schiefer AI, Egger G (2013) Combating the epigenome: epigenetic drugs against non-Hodgkin's lymphoma. Epigenomics 5(4):397–415. doi:10.2217/epi.13.39
28. Pasqualucci L, Dominguez-Sola D, Chiarenza A, Fabbri G, Grunn A, Trifonov V, Kasper LH, Lerach S, Tang H, Ma J, Rossi D, Chadburn A, Murty VV, Mullighan CG, Gaidano G, Rabadan R, Brindle PK, Dalla-Favera R (2011) Inactivating mutations of acetyltransferase genes in B-cell lymphoma. Nature 471(7337):189–195. doi:10.1038/nature09730
29. Dawson MA, Kouzarides T (2012) Cancer epigenetics: from mechanism to therapy. Cell 150(1):12–27. doi:10.1016/j.cell.2012.06.013
30. Witt O, Deubzer HE, Milde T, Oehme I (2009) HDAC family: what are the cancer relevant targets? Cancer Lett 277(1):8–21. doi:10.1016/j.canlet.2008.08.016
31. Kim HJ, Bae SC (2011) Histone deacetylase inhibitors: molecular mechanisms of action and clinical trials as anti-cancer drugs. Am J Transl Res 3(2):166–179
32. Kristensen LS, Nielsen HM, Hansen LL (2009) Epigenetics and cancer treatment. Eur J Pharmacol 625(1–3):131–142. doi:10.1016/j.ejphar.2009.10.011
33. West AC, Johnstone RW (2014) New and emerging HDAC inhibitors for cancer treatment. J Clin Invest 124(1):30–39. doi:10.1172/JCI69738
34. Min SK, Koh YH, Park Y, Kim HJ, Seo J, Park HR, Cho SJ, Kim IS (2012) Expression of HAT1 and HDAC1, 2, 3 in diffuse large B-cell lymphomas, peripheral T-cell lymphomas, and NK/T-cell lymphomas. Korean J Pathol 46(2):142–150. doi:10.4132/KoreanJPathol.2012.46.2.142
35. Heideman MR, Wilting RH, Yanover E, Velds A, de Jong J, Kerkhoven RM, Jacobs H, Wessels LF, Dannenberg JH (2013) Dosage-dependent tumor suppression by histone deacetylases 1 and 2 through regulation of c-Myc collaborating genes and p53 function. Blood 121(11):2038–2050. doi:10.1182/blood-2012-08-450916
36. Lee SH, Yoo C, Im S, Jung JH, Choi HJ, Yoo J (2014) Expression of histone deacetylases in diffuse large B-cell lymphoma and its clinical significance. Int J Med Sci 11(10):994–1000. doi:10.7150/ijms.8522
37. Gruhn B, Naumann T, Gruner D, Walther M, Wittig S, Becker S, Beck JF, Sonnemann J (2013) The expression of histone deacetylase 4 is associated with prednisone poor-response in childhood acute lymphoblastic leukemia. Leuk Res 37(10):1200–1207. doi:10.1016/j.leukres.2013.07.016
38. New M, Olzscha H, La Thangue NB (2012) HDAC inhibitor-based therapies: can we interpret the code? Mol Oncol 6(6):637–656. doi:10.1016/j.molonc.2012.09.003
39. Seto E, Yoshida M (2014) Erasers of histone acetylation: the histone deacetylase enzymes. Cold Spring Harb Perspect Biol 6(4):a018713. doi:10.1101/cshperspect.a018713
40. Heltweg B, Gatbonton T, Schuler AD, Posakony J, Li H, Goehle S, Kollipara R, Depinho RA, Gu Y, Simon JA, Bedalov A (2006) Antitumor activity of a small-molecule inhibitor of human silent information regulator 2 enzymes. Cancer Res 66(8):4368–4377. doi:10.1158/0008-5472.CAN-05-3617
41. Ropero S, Esteller M (2007) The role of histone deacetylases (HDACs) in human cancer. Mol Oncol 1(1):19–25. doi:10.1016/j.molonc.2007.01.001
42. Glozak MA, Seto E (2009) Acetylation/deacetylation modulates the stability of DNA replication licensing factor Cdt1. J Biol Chem 284(17):11446–11453. doi:10.1074/jbc.M809394200
43. Witt O, Deubzer HE, Lodrini M, Milde T, Oehme I (2009) Targeting histone deacetylases in neuroblastoma. Curr Pharm Des 15(4):436–447

44. Caron C, Boyault C, Khochbin S (2005) Regulatory cross-talk between lysine acetylation and ubiquitination: role in the control of protein stability. Bioessays 27(4):408–415. doi:10.1002/bies.20210
45. Choudhary C, Kumar C, Gnad F, Nielsen ML, Rehman M, Walther TC, Olsen JV, Mann M (2009) Lysine acetylation targets protein complexes and co-regulates major cellular functions. Science 325(5942):834–840. doi:10.1126/science.1175371
46. Bereshchenko OR, Gu W, Dalla-Favera R (2002) Acetylation inactivates the transcriptional repressor BCL6. Nat Genet 32(4):606–613. doi:10.1038/ng1018
47. Gu W, Roeder RG (1997) Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. Cell 90(4):595–606
48. Matsuyama A, Shimazu T, Sumida Y, Saito A, Yoshimatsu Y, Seigneurin-Berny D, Osada H, Komatsu Y, Nishino N, Khochbin S, Horinouchi S, Yoshida M (2002) In vivo destabilization of dynamic microtubules by HDAC6-mediated deacetylation. EMBO J 21(24):6820–6831
49. Grignani F, De Matteis S, Nervi C, Tomassoni L, Gelmetti V, Cioce M, Fanelli M, Ruthardt M, Ferrara FF, Zamir I, Seiser C, Lazar MA, Minucci S, Pelicci PG (1998) Fusion proteins of the retinoic acid receptor-alpha recruit histone deacetylase in promyelocytic leukaemia. Nature 391(6669):815–818. doi:10.1038/35901
50. Cress WD, Seto E (2000) Histone deacetylases, transcriptional control, and cancer. J Cell Physiol 184(1):1–16. doi:10.1002/(SICI)1097-4652(200007)184:1<1::AID-JCP1>3.0.CO;2–7
51. Yang H, Maddipoti S, Quesada A, Bohannan Z, Cabrero Calvo M, Colla S, Wei Y, Estecio M, Wierda W, Bueso-Ramos C, Garcia-Manero G (2015) Analysis of class I and II histone deacetylase gene expression in human leukemia. Leuk Lymphoma 26:1–8. doi:10.3109/10428194.2015.1034705
52. Chun P (2015) Histone deacetylase inhibitors in hematological malignancies and solid tumors. Arch Pharm Res 38(6):933–949. doi:10.1007/s12272-015-0571-1
53. Riggs MG, Whittaker RG, Neumann JR, Ingram VM (1977) n-Butyrate causes histone modification in HeLa and Friend erythroleukaemia cells. Nature 268(5619):462–464
54. Grant S, Easley C, Kirkpatrick P (2007) Vorinostat. Nat Rev Drug Discov 6(1):21–22. doi:10.1038/nrd2227
55. Bolden JE, Peart MJ, Johnstone RW (2006) Anticancer activities of histone deacetylase inhibitors. Nat Rev Drug Discov 5(9):769–784. doi:10.1038/nrd2133
56. Richon VM, Emiliani S, Verdin E, Webb Y, Breslow R, Rifkind RA, Marks PA (1998) A class of hybrid polar inducers of transformed cell differentiation inhibits histone deacetylases. Proc Natl Acad Sci U S A 95(6):3003–3007
57. Kirschbaum M, Frankel P, Popplewell L, Zain J, Delioukina M, Pullarkat V, Matsuoka D, Pulone B, Rotter AJ, Espinoza-Delgado I, Nademanee A, Forman SJ, Gandara D, Newman E (2011) Phase II study of vorinostat for treatment of relapsed or refractory indolent non-Hodgkin's lymphoma and mantle cell lymphoma. J Clin Oncol 29(9):1198–1203. doi:10.1200/JCO.2010.32.1398
58. Ogura M, Ando K, Suzuki T, Ishizawa K, Oh SY, Itoh K, Yamamoto K, Au WY, Tien HF, Matsuno Y, Terauchi T, Mori M, Tanaka Y, Shimamoto T, Tobinai K, Kim WS (2014) A multicentre phase II study of vorinostat in patients with relapsed or refractory indolent B-cell non-Hodgkin lymphoma and mantle cell lymphoma. Br J Haematol 165(6):768–776. doi:10.1111/bjh.12819
59. Nakajima H, Kim YB, Terano H, Yoshida M, Horinouchi S (1998) FR901228, a potent antitumor antibiotic, is a novel histone deacetylase inhibitor. Exp Cell Res 241(1):126–133. doi:10.1006/excr.1998.4027
60. Byrd JC, Marcucci G, Parthun MR, Xiao JJ, Klisovic RB, Moran M, Lin TS, Liu S, Sklenar AR, Davis ME, Lucas DM, Fischer B, Shank R, Tejaswi SL, Binkley P, Wright J, Chan KK, Grever MR (2005) A phase 1 and pharmacodynamic study of depsipeptide (FK228) in chronic lymphocytic leukemia and acute myeloid leukemia. Blood 105(3):959–967. doi:10.1182/blood-2004-05-1693
61. Klimek VM, Fircanis S, Maslak P, Guernah I, Baum M, Wu N, Panageas K, Wright JJ, Pandolfi PP, Nimer SD (2008) Tolerability, pharmacodynamics, and pharmacokinetics studies of depsipeptide (romidepsin) in patients with acute myelogenous leukemia or advanced myelodysplastic syndromes. Clin Cancer Res 14(3):826–832. doi:10.1158/1078-0432.CCR-07-0318
62. Piekarz RL, Frye R, Turner M, Wright JJ, Allen SL, Kirschbaum MH, Zain J, Prince HM, Leonard JP, Geskin LJ, Reeder C, Joske D, Figg WD, Gardner ER, Steinberg SM, Jaffe ES, Stetler-Stevenson M, Lade S, Fojo AT, Bates SE (2009) Phase II multi-institutional trial of the histone deacetylase inhibitor romidepsin as monotherapy for patients with cutaneous T-cell lymphoma. J Clin Oncol 27(32):5410–5417. doi:10.1200/JCO.2008.21.6150
63. Bates SE, Zhan Z, Steadman K, Obrzut T, Luchenko V, Frye R, Robey RW, Turner M, Gardner ER, Figg WD, Steinberg SM, Ling A, Fojo T, To KW, Piekarz RL (2010) Laboratory correlates for a phase II trial of romidepsin in cutaneous and peripheral T-cell lymphoma. Br J Haematol 148(2):256–267. doi:10.1111/j.1365-2141.2009.07954.x
64. Gimsing P (2009) Belinostat: a new broad acting antineoplastic histone deacetylase inhibitor. Expert Opin Investig Drugs 18(4):501–508. doi:10.1517/13543780902852560
65. Zain JM, O'Connor O (2010) Targeted treatment and new agents in peripheral T-cell lymphoma. Int J Hematol 92(1):33–44. doi:10.1007/s12185-010-0614-9
66. Foss F, Advani R, Duvic M, Hymes KB, Intragumtornchai T, Lekhakula A, Shpilberg O, Lerner A, Belt RJ, Jacobsen ED, Laurent G, Ben-Yehuda D, Beylot-Barry M, Hillen U, Knoblauch P, Bhat G, Chawla S, Allen LF, Pohlman B (2015) A Phase II trial of Belinostat (PXD101) in patients with relapsed or refractory peripheral or cutaneous T-cell lymphoma. Br J Haematol 168(6):811–819. doi:10.1111/bjh.13222
67. De Souza C, Chatterji BP (2015) HDAC inhibitors as novel anticancer therapeutics. Recent Pat Anticancer Drug Discov 10(2):145–162
68. Gong K, Xie J, Yi H, Li W (2012) CS055 (Chidamide/HBI-8000), a novel histone deacetylase inhibitor, induces G1 arrest, ROS-dependent apoptosis and differentiation in human leukaemia cells. Biochem J 443(3):735–746. doi:10.1042/BJ20111685
69. Dong M, Ning ZQ, Xing PY, Xu JL, Cao HX, Dou GF, Meng ZY, Shi YK, Lu XP, Feng FY (2012) Phase I study of chidamide (CS055/HBI-8000), a new histone deacetylase inhibitor, in patients with advanced solid tumors and lymphomas. Cancer Chemother Pharmacol 69(6):1413–1422. doi:10.1007/s00280-012-1847-5
70. Rambaldi A, Dellacasa CM, Finazzi G, Carobbio A, Ferrari ML, Guglielmelli P, Gattoni E, Salmoiraghi S, Finazzi MC, Di Tollo S, D'Urzo C, Vannucchi AM, Barosi G, Barbui T (2010) A pilot study of the histone-deacetylase inhibitor Givinostat in patients with JAK2V617F positive chronic myeloproliferative neoplasms. Br J Haematol 150(4):446–455. doi:10.1111/j.1365-2141.2010.08266.x
71. Duvic M, Dummer R, Becker JC, Poulalhon N, Ortiz Romero P, Grazia Bernengo M, Lebbe C, Assaf C, Squier M, Williams D, Marshood M, Tai F, Prince HM (2013) Panobinostat activity in both bexarotene-exposed and -naive patients with refractory cutaneous T-cell lymphoma: results of a phase II trial. Eur J Cancer 49(2):386–394. doi:10.1016/j.ejca.2012.08.017
72. Dickinson M, Ritchie D, DeAngelo DJ, Spencer A, Ottmann OG, Fischer T, Bhalla KN, Liu A, Parker K, Scott JW, Bishton M,

Prince HM (2009) Preliminary evidence of disease response to the pan deacetylase inhibitor panobinostat (LBH589) in refractory Hodgkin lymphoma. Br J Haematol 147(1):97–101. doi:10.1111/j.1365-2141.2009.07837.x

73. DeAngelo DJ, Spencer A, Bhalla KN, Prince HM, Fischer T, Kindler T, Giles FJ, Scott JW, Parker K, Liu A, Woo M, Atadja P, Mishra KK, Ottmann OG (2013) Phase Ia/II, two-arm, open-label, dose-escalation study of oral panobinostat administered via two dosing schedules in patients with advanced hematologic malignancies. Leukemia 27(8):1628–1636. doi:10.1038/leu.2013.38
74. Rao R, Balusu R, Fiskus W, Mudunuru U, Venkannagari S, Chauhan L, Smith JE, Hembruff SL, Ha K, Atadja P, Bhalla KN (2012) Combination of pan-histone deacetylase inhibitor and autophagy inhibitor exerts superior efficacy against triple-negative human breast cancer cells. Mol Cancer Ther 11(4):973–983. doi:10.1158/1535-7163.MCT-11-0979
75. Chateauvieux S, Morceau F, Dicato M, Diederich M (2010) Molecular and therapeutic potential and toxicity of valproic acid. J Biomed Biotechnol. doi:10.1155/2010/479364
76. Bokelmann I, Mahlknecht U (2008) Valproic acid sensitizes chronic lymphocytic leukemia cells to apoptosis and restores the balance between pro- and antiapoptotic proteins. Mol Med 14(1–2):20–27. doi:10.2119/2007-00084.Bokelmann
77. Saito A, Yamashita T, Mariko Y, Nosaka Y, Tsuchiya K, Ando T, Suzuki T, Tsuruo T, Nakanishi O (1999) A synthetic inhibitor of histone deacetylase, MS-27-275, with marked in vivo antitumor activity against human tumors. Proc Natl Acad Sci U S A 96(8):4592–4597
78. Pili R, Salumbides B, Zhao M, Altiok S, Qian D, Zwiebel J, Carducci MA, Rudek MA (2012) Phase I study of the histone deacetylase inhibitor entinostat in combination with 13-cis retinoic acid in patients with solid tumours. Br J Cancer 106(1):77–84. doi:10.1038/bjc.2011.527
79. Bonfils C, Kalita A, Dubay M, Siu LL, Carducci MA, Reid G, Martell RE, Besterman JM, Li Z (2008) Evaluation of the pharmacodynamic effects of MGCD0103 from preclinical models to human using a novel HDAC enzyme assay. Clin Cancer Res 14(11):3441–3449. doi:10.1158/1078-0432.CCR-07-4427
80. Boumber Y, Younes A, Garcia-Manero G (2011) Mocetinostat (MGCD0103): a review of an isotype-specific histone deacetylase inhibitor. Expert Opin Investig Drugs 20(6):823–829. doi:10.1517/13543784.2011.577737
81. Siu LL, Pili R, Duran I, Messersmith WA, Chen EX, Sullivan R, MacLean M, King S, Brown S, Reid GK, Li Z, Kalita AM, Laille EJ, Besterman JM, Martell RE, Carducci MA (2008) Phase I study of MGCD0103 given as a three-times-per-week oral dose in patients with advanced solid tumors. J Clin Oncol 26(12):1940–1947. doi:10.1200/JCO.2007.14.5730
82. Rivera-Del Valle N, Gao S, Miller CP, Fulbright J, Gonzales C, Sirisawad M, Steggerda S, Wheler J, Balasubramanian S, Chandra J (2010) PCI-24781, a novel hydroxamic acid HDAC inhibitor, exerts cytotoxicity and histone alterations via caspase-8 and FADD in leukemia cells. Int J Cell Biol 2010:207420. doi:10.1155/2010/207420
83. Morschhauser F, Terriou L, Coiffier B, Bachy E, Varga A, Kloos I, Lelievre H, Sarry AL, Depil S, Ribrag V (2015) Phase 1 study of the oral histone deacetylase inhibitor abexinostat in patients with Hodgkin lymphoma, non-Hodgkin lymphoma, or chronic lymphocytic leukaemia. Investig New Drugs 33(2):423–431. doi:10.1007/s10637-015-0206-x
84. Haggarty SJ, Koeller KM, Wong JC, Grozinger CM, Schreiber SL (2003) Domain-selective small-molecule inhibitor of histone deacetylase 6 (HDAC6)-mediated tubulin deacetylation. Proc Natl Acad Sci U S A 100(8):4389–4394. doi:10.1073/pnas.0430973100
85. Balasubramanian S, Ramos J, Luo W, Sirisawad M, Verner E, Buggy JJ (2008) A novel histone deacetylase 8 (HDAC8)-specific inhibitor PCI-34051 induces apoptosis in T-cell lymphomas. Leukemia 22(5):1026–1034. doi:10.1038/leu.2008.9
86. Methot JL, Chakravarty PK, Chenard M, Close J, Cruz JC, Dahlberg WK, Fleming J, Hamblett CL, Hamill JE, Harrington P, Harsch A, Heidebrecht R, Hughes B, Jung J, Kenific CM, Kral AM, Meinke PT, Middleton RE, Ozerova N, Sloman DL, Stanton MG, Szewczak AA, Tyagarajan S, Witter DJ, Secrist JP, Miller TA (2008) Exploration of the internal cavity of histone deacetylase (HDAC) with selective HDAC1/HDAC2 inhibitors (SHI-1:2). Bioorg Med Chem Lett 18(3):973–978. doi:10.1016/j.bmcl.2007.12.031
87. Thurn KT, Thomas S, Moore A, Munster PN (2011) Rational therapeutic combinations with histone deacetylase inhibitors for the treatment of cancer. Future Oncol 7(2):263–283. doi:10.2217/fon.11.2
88. Li L, Wang L, Wang Z, Ho Y, McDonald T, Holyoake TL, Chen W, Bhatia R (2012) Activation of p53 by SIRT1 inhibition enhances elimination of CML leukemia stem cells in combination with imatinib. Cancer Cell 21(2):266–281. doi:10.1016/j.ccr.2011.12.020
89. Insinga A, Monestiroli S, Ronzoni S, Gelmetti V, Marchesi F, Viale A, Altucci L, Nervi C, Minucci S, Pelicci PG (2005) Inhibitors of histone deacetylases induce tumor-selective apoptosis through activation of the death receptor pathway. Nat Med 11(1):71–76. doi:10.1038/nm1160
90. Dalla-Favera R, Bregni M, Erikson J, Patterson D, Gallo RC, Croce CM (1982) Human c-myc onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. Proc Natl Acad Sci U S A 79(24):7824–7827
91. Taub R, Kirsch I, Morton C, Lenoir G, Swan D, Tronick S, Aaronson S, Leder P (1982) Translocation of the c-myc gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. Proc Natl Acad Sci U S A 79(24):7837–7841
92. Bakhshi A, Jensen JP, Goldman P, Wright JJ, McBride OW, Epstein AL, Korsmeyer SJ (1985) Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around JH on chromosome 14 and near a transcriptional unit on 18. Cell 41(3):899–906
93. Cleary ML, Sklar J (1985) Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint-cluster region near a transcriptionally active locus on chromosome 18. Proc Natl Acad Sci U S A 82(21):7439–7443
94. Liu H, Wang J, Epner EM (2004) Cyclin D1 activation in B-cell malignancy: association with changes in histone acetylation, DNA methylation, and RNA polymerase II binding to both promoter and distal sequences. Blood 104(8):2505–2513. doi:10.1182/blood-2004-02-0483
95. Singh BN, Zhang G, Hwa YL, Li J, Dowdy SC, Jiang S-W (2010) Nonhistone protein acetylation as cancer therapy targets. Expert Rev Anticancer Ther 10(6):935–954. doi:10.1586/era.10.62
96. Glozak MA, Sengupta N, Zhang X, Seto E (2005) Acetylation and deacetylation of non-histone proteins. Gene 363:15–23. doi:10.1016/j.gene.2005.09.010
97. Dokmanovic M, Clarke C, Marks PA (2007) Histone deacetylase inhibitors: overview and perspectives. Mol Cancer Res 5(10):981–989. doi:10.1158/1541-7786.MCR-07-0324
98. Eot-Houllier G, Fulcrand G, Magnaghi-Jaulin L, Jaulin C (2009) Histone deacetylase inhibitors and genomic instability. Cancer Lett 274(2):169–176. doi:10.1016/j.canlet.2008.06.005
99. Grant C, Rahman F, Piekarz R, Peer C, Frye R, Robey RW, Gardner ER, Figg WD, Bates SE (2010) Romidepsin: a new therapy for cutaneous T-cell lymphoma and a potential therapy for

solid tumors. Expert Rev Anticancer Ther 10(7):997–1008. doi:10.1586/era.10.88

100. Cotto M, Cabanillas F, Tirado M, Garcia MV, Pacheco E (2010) Epigenetic therapy of lymphoma using histone deacetylase inhibitors. Clin Transl Oncol 12(6):401–409. doi:10.1007/s12094-010-0527-3
101. Batova A, Shao LE, Diccianni MB, Yu AL, Tanaka T, Rephaeli A, Nudelman A, Yu J (2002) The histone deacetylase inhibitor AN-9 has selective toxicity to acute leukemia and drug-resistant primary leukemia and cancer cell lines. Blood 100(9):3319–3324. doi:10.1182/blood-2002-02-0567
102. Cortez CC, Jones PA (2008) Chromatin, cancer and drug therapies. Mutat Res 647(1–2):44–51. doi:10.1016/j.mrfmmm.2008.07.006
103. Garcia-Manero G, Assouline S, Cortes J, Estrov Z, Kantarjian H, Yang H, Newsome WM, Miller WH Jr, Rousseau C, Kalita A, Bonfils C, Dubay M, Patterson TA, Li Z, Besterman JM, Reid G, Laille E, Martell RE, Minden M (2008) Phase 1 study of the oral isotype specific histone deacetylase inhibitor MGCD0103 in leukemia. Blood 112(4):981–989. doi:10.1182/blood-2007-10-115873
104. Jin Y, Cao Q, Chen C, Du X, Jin B, Pan J (2015) Tenovin-6-mediated inhibition of SIRT1/2 induces apoptosis in acute lymphoblastic leukemia (ALL) cells and eliminates ALL stem/progenitor cells. BMC Cancer 15:226. doi:10.1186/s12885-015-1282-1
105. Liu HL, Chen Y, Cui GH, Zhou JF (2005) Curcumin, a potent anti-tumor reagent, is a novel histone deacetylase inhibitor regulating B-NHL cell line Raji proliferation. Acta Pharmacol Sin 26(5):603–609. doi:10.1111/j.1745-7254.2005.00081.x

BIBLIOGRAPHY

Agrelo, R., Setien, F., Espada, J., Artiga, M.J., Rodriguez, M., Perez-Rosado, A., Sanchez-Aguilera, A., Fraga, M.F., Piris, M.A., and Esteller, M. (2005). Inactivation of the lamin A/C gene by CpG island promoter hypermethylation in hematologic malignancies, and its association with poor survival in nodal diffuse large B-cell lymphoma. J Clin Oncol *23*, 3940-3947.

Ahn, S.H., Cheung, W.L., Hsu, J.Y., Diaz, R.L., Smith, M.M., and Allis, C.D. (2005). Sterile 20 kinase phosphorylates histone H2B at serine 10 during hydrogen peroxide-induced apoptosis in S. cerevisiae. Cell *120*, 25-36.

Ait-Si-Ali, S., Ramirez, S., Barre, F.X., Dkhissi, F., Magnaghi-Jaulin, L., Girault, J.A., Robin, P., Knibiehler, M., Pritchard, L.L., Ducommun, B.*, et al.* (1998). Histone acetyltransferase activity of CBP is controlled by cycle-dependent kinases and oncoprotein E1A. Nature *396*, 184-186.

Aitman, T.J., Dong, R., Vyse, T.J., Norsworthy, P.J., Johnson, M.D., Smith, J., Mangion, J., Roberton-Lowe, C., Marshall, A.J., Petretto, E.*, et al.* (2006). Copy number polymorphism in Fcgr3 predisposes to glomerulonephritis in rats and humans. Nature *439*, 851-855.

Alao, J.P. (2007). The regulation of cyclin D1 degradation: roles in cancer development and the potential for therapeutic invention. Mol Cancer *6*, 24.

Alao, J.P., Lam, E.W., Ali, S., Buluwela, L., Bordogna, W., Lockey, P., Varshochi, R., Stavropoulou, A.V., Coombes, R.C., and Vigushin, D.M. (2004). Histone deacetylase inhibitor trichostatin A represses estrogen receptor alpha-dependent transcription and promotes proteasomal degradation of cyclin D1 in human breast carcinoma cell lines. Clin Cancer Res *10*, 8094-8104.

Alao, J.P., Stavropoulou, A.V., Lam, E.W., Coombes, R.C., and Vigushin, D.M. (2006). Histone deacetylase inhibitor, trichostatin A induces ubiquitin-dependent cyclin D1 degradation in MCF-7 breast cancer cells. Mol Cancer *5*, 8.

Albert, M., and Helin, K. (2010). Histone methyltransferases in cancer. Semin Cell Dev Biol *21*, 209-220.

Albiez, H., Cremer, M., Tiberi, C., Vecchio, L., Schermelleh, L., Dittrich, S., Kupper, K., Joffe, B., Thormeyer, T., von Hase, J.*, et al.* (2006). Chromatin domains and the interchromatin compartment form structurally defined and functionally interacting nuclear networks. Chromosome Res *14*, 707-733.

Allinne, J., Pichugin, A., Iarovaia, O., Klibi, M., Barat, A., Zlotek-Zlotkiewicz, E., Markozashvili, D., Petrova, N., Camara-Clayette, V., Ioudinkova, E.*, et al.* (2014). Perinucleolar relocalization and nucleolin as crucial events in the transcriptional activation of key genes in mantle cell lymphoma. Blood *123*, 2044-2053.

Alt, F.W., Zhang, Y., Meng, F.L., Guo, C., and Schwer, B. (2013). Mechanisms of programmed DNA lesions and genomic instability in the immune system. Cell *152*, 417-429.

Amara, K., Ziadi, S., Hachana, M., Soltani, N., Korbi, S., and Trimeche, M. (2010). DNA methyltransferase DNMT3b protein overexpression as a prognostic factor in patients with diffuse large B-cell lymphomas. Cancer Sci *101*, 1722-1730.

Artandi, S.E., Chang, S., Lee, S.L., Alson, S., Gottlieb, G.J., Chin, L., and DePinho, R.A. (2000). Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. Nature *406*, 641-645.

Avvakumov, N., and Cote, J. (2007). The MYST family of histone acetyltransferases and their intimate links to cancer. Oncogene *26*, 5395-5407.

Baek, S.H., Ohgi, K.A., Rose, D.W., Koo, E.H., Glass, C.K., and Rosenfeld, M.G. (2002). Exchange of N-CoR corepressor and Tip60 coactivator complexes links gene expression by NF-kappaB and beta-amyloid precursor protein. Cell *110*, 55-67.

Bakhshi, A., Jensen, J.P., Goldman, P., Wright, J.J., McBride, O.W., Epstein, A.L., and Korsmeyer, S.J. (1985). Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around JH on chromosome 14 and near a transcriptional unit on 18. Cell *41*, 899-906.

Balasubramanian, S., Ramos, J., Luo, W., Sirisawad, M., Verner, E., and Buggy, J.J. (2008). A novel histone deacetylase 8 (HDAC8)-specific inhibitor PCI-34051 induces apoptosis in T-cell lymphomas. Leukemia *22*, 1026-1034.

Bannister, A.J., and Kouzarides, T. (2011). Regulation of chromatin by histone modifications. Cell Res *21*, 381-395.

Barber, C.M., Turner, F.B., Wang, Y., Hagstrom, K., Taverna, S.D., Mollah, S., Ueberheide, B., Meyer, B.J., Hunt, D.F., Cheung, P.*, et al.* (2004). The enhancement of histone H4 and H2A serine 1 phosphorylation during mitosis and S-phase is evolutionarily conserved. Chromosoma *112*, 360-371.

Barski, A., Cuddapah, S., Cui, K., Roh, T.Y., Schones, D.E., Wang, Z., Wei, G., Chepelev, I., and Zhao, K. (2007). High-resolution profiling of histone methylations in the human genome. Cell *129*, 823-837.

Bartova, E., and Kozubek, S. (2006). Nuclear architecture in the light of gene expression and cell differentiation studies. Biol Cell *98*, 323-336.

Bates, S.E., Zhan, Z., Steadman, K., Obrzut, T., Luchenko, V., Frye, R., Robey, R.W., Turner, M., Gardner, E.R., Figg, W.D.*, et al.* (2010). Laboratory correlates for a phase II trial of romidepsin in cutaneous and peripheral T-cell lymphoma. Br J Haematol *148*, 256-267.

Batova, A., Shao, L.E., Diccianni, M.B., Yu, A.L., Tanaka, T., Rephaeli, A., Nudelman, A., and Yu, J. (2002). The histone deacetylase inhibitor AN-9 has selective toxicity to acute leukemia and drug-resistant primary leukemia and cancer cell lines. Blood *100*, 3319-3324.

Becker, P.B., Ruppert, S., and Schutz, G. (1987). Genomic footprinting reveals cell type-specific DNA binding of ubiquitous factors. Cell *51*, 435-443.

Bedford, M.T., and Clarke, S.G. (2009). Protein arginine methylation in mammals: who, what, and why. Mol Cell *33*, 1-13.

Bender, C.M., Pao, M.M., and Jones, P.A. (1998). Inhibition of DNA methylation by 5-aza-2'-deoxycytidine suppresses the growth of human tumor cell lines. Cancer Res *58*, 95-101.

Bennaceur-Griscelli, A., Bosq, J., Koscielny, S., Lefrere, F., Turhan, A., Brousse, N., Hermine, O., and Ribrag, V. (2004). High level of glutathione-S-transferase pi expression in mantle cell lymphomas. Clin Cancer Res *10*, 3029-3034.

Bereshchenko, O.R., Gu, W., and Dalla-Favera, R. (2002). Acetylation inactivates the transcriptional repressor BCL6. Nat Genet *32*, 606-613.

Bergman, Y., and Cedar, H. (2013). DNA methylation dynamics in health and disease. Nat Struct Mol Biol *20*, 274-281.

Bernstein, B.E., Meissner, A., and Lander, E.S. (2007). The mammalian epigenome. Cell *128*, 669-681.

Bertoni, F., Zucca, E., and Cavalli, F. (2004). Mantle cell lymphoma. Curr Opin Hematol *11*, 411-418.

Bird, A. (2002). DNA methylation patterns and epigenetic memory. Genes Dev *16*, 6-21.

Bodnar, J.W. (1988). A domain model for eukaryotic DNA organization: a molecular basis for cell differentiation and chromosome evolution. J Theor Biol *132*, 479-507.

Bokelmann, I., and Mahlknecht, U. (2008). Valproic acid sensitizes chronic lymphocytic leukemia cells to apoptosis and restores the balance between pro- and antiapoptotic proteins. Mol Med *14*, 20-27.

Bolden, J.E., Peart, M.J., and Johnstone, R.W. (2006). Anticancer activities of histone deacetylase inhibitors. Nat Rev Drug Discov *5*, 769-784.

Bolzer, A., Kreth, G., Solovei, I., Koehler, D., Saracoglu, K., Fauth, C., Muller, S., Eils, R., Cremer, C., Speicher, M.R.*, et al.* (2005). Three-dimensional maps of all chromosomes in human male fibroblast nuclei and prometaphase rosettes. PLoS Biol *3*, e157.

Bonfils, C., Kalita, A., Dubay, M., Siu, L.L., Carducci, M.A., Reid, G., Martell, R.E., Besterman, J.M., and Li, Z. (2008). Evaluation of the pharmacodynamic effects of MGCD0103 from preclinical models to human using a novel HDAC enzyme assay. Clin Cancer Res *14*, 3441-3449.

Bosch, F., Jares, P., Campo, E., Lopez-Guillermo, A., Piris, M.A., Villamor, N., Tassies, D., Jaffe, E.S., Montserrat, E., Rozman, C.*, et al.* (1994). PRAD-1/cyclin D1 gene overexpression in chronic lymphoproliferative disorders: a highly specific marker of mantle cell lymphoma. Blood *84*, 2726-2732.

Boveri, T. (1914). Zur Frage der Entstehung maligner Tumoren (G. Fischer).

Burman, B., Zhang, Z.Z., Pegoraro, G., Lieb, J.D., and Misteli, T. (2015). Histone modifications predispose genome regions to breakage and translocation. Genes Dev *29*, 1393-1402.

Byrd, J.C., Marcucci, G., Parthun, M.R., Xiao, J.J., Klisovic, R.B., Moran, M., Lin, T.S., Liu, S., Sklenar, A.R., Davis, M.E.*, et al.* (2005). A phase 1 and pharmacodynamic study of depsipeptide (FK228) in chronic lymphocytic leukemia and acute myeloid leukemia. Blood *105*, 959-967.

Cagnard, N., C. Lucchesi, and G. Chiocchia (2009). GExMap: An Intuitive Visual Tool to Detect and Analyze Genomic Distribution in Microarraygenerated Lists of Differentially Expressed Genes. Proteomics Bioinform *2*, 51-59.

Calin, G.A., Liu, C.G., Ferracin, M., Hyslop, T., Spizzo, R., Sevignani, C., Fabbri, M., Cimmino, A., Lee, E.J., Wojcik, S.E.*, et al.* (2007). Ultraconserved regions encoding ncRNAs are altered in human leukemias and carcinomas. Cancer Cell *12*, 215-229.

Camara-Clayette, V., Hermine, O., and Ribrag, V. (2012). Emerging agents for the treatment of mantle cell lymphoma. Expert Rev Anticancer Ther *12*, 1205-1215.

Cameron, E.E., Bachman, K.E., Myohanen, S., Herman, J.G., and Baylin, S.B. (1999). Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat Genet *21*, 103-107.

Cappuzzo, F., Hirsch, F.R., Rossi, E., Bartolini, S., Ceresoli, G.L., Bemis, L., Haney, J., Witta, S., Danenberg, K., Domenichini, I.*, et al.* (2005). Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. J Natl Cancer Inst *97*, 643-655.

Caron, C., Boyault, C., and Khochbin, S. (2005). Regulatory cross-talk between lysine acetylation and ubiquitination: role in the control of protein stability. Bioessays *27*, 408-415.

Carthew, R.W., and Sontheimer, E.J. (2009). Origins and Mechanisms of miRNAs and siRNAs. Cell *136*, 642-655.

Castanotto, D., Tommasi, S., Li, M., Li, H., Yanow, S., Pfeifer, G.P., and Rossi, J.J. (2005). Short hairpin RNA-directed cytosine (CpG) methylation of the RASSF1A gene promoter in HeLa cells. Mol Ther *12*, 179-183.

Cerchietti, L.C., Hatzi, K., Caldas-Lopes, E., Yang, S.N., Figueroa, M.E., Morin, R.D., Hirst, M., Mendez, L., Shaknovich, R., Cole, P.A.*, et al.* (2010). BCL6 repression of EP300 in human diffuse large B cell lymphoma cells provides a basis for rational combinatorial therapy. J Clin Invest.

Chan, J.A., Krichevsky, A.M., and Kosik, K.S. (2005). MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. Cancer Res *65*, 6029-6033.

Chang, B., Chen, Y., Zhao, Y., and Bruick, R.K. (2007). JMJD6 is a histone arginine demethylase. Science *318*, 444-447.

Cheminant, M., Robinson, S., Ribrag, V., Le Gouill, S., Suarez, F., Delarue, R., and Hermine, O. (2015). Prognosis and outcome of stem cell transplantation for mantle cell lymphoma. Expert Rev Hematol *8*, 493-504.

Chen, J., Young, F., Bottaro, A., Stewart, V., Smith, R.K., and Alt, F.W. (1993). Mutations of the intronic IgH enhancer and its flanking sequences differentially affect accessibility of the JH locus. EMBO J *12*, 4635-4645.

Chen, Z., Place, R.F., Jia, Z.J., Pookot, D., Dahiya, R., and Li, L.C. (2008). Antitumor effect of dsRNA-induced p21(WAF1/CIP1) gene activation in human bladder cancer cells. Mol Cancer Ther *7*, 698-703.

Chi, P., Allis, C.D., and Wang, G.G. (2010). Covalent histone modifications--miswritten, misinterpreted and mis-erased in human cancers. Nat Rev Cancer *10*, 457-469.

Choudhary, C., Kumar, C., Gnad, F., Nielsen, M.L., Rehman, M., Walther, T.C., Olsen, J.V., and Mann, M. (2009). Lysine acetylation targets protein complexes and co-regulates major cellular functions. Science *325*, 834-840.

Chowdhury, S., Erickson, S.W., MacLeod, S.L., Cleves, M.A., Hu, P., Karim, M.A., and Hobbs, C.A. (2011). Maternal genome-wide DNA methylation patterns and congenital heart defects. PLoS One *6*, e16506.

Christophorou, M.A., Castelo-Branco, G., Halley-Stott, R.P., Oliveira, C.S., Loos, R., Radzisheuskaya, A., Mowen, K.A., Bertone, P., Silva, J.C., Zernicka-Goetz, M.*, et al.* (2014). Citrullination regulates pluripotency and histone H1 binding to chromatin. Nature *507*, 104-108.

Chuang, C.H., Carpenter, A.E., Fuchsova, B., Johnson, T., de Lanerolle, P., and Belmont, A.S. (2006). Long-range directional movement of an interphase chromosome site. Curr Biol *16*, 825-831.

Chun, P. (2015). Histone deacetylase inhibitors in hematological malignancies and solid tumors. Arch Pharm Res *38*, 933-949.

Cihak, A. (1974). Biological effects of 5-azacytidine in eukaryotes. Oncology *30*, 405-422.

Cleary, M.L., and Sklar, J. (1985). Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint-cluster region near a transcriptionally active locus on chromosome 18. Proc Natl Acad Sci U S A *82*, 7439-7443.

Clissold, P.M., and Ponting, C.P. (2001). JmjC: cupin metalloenzyme-like domains in jumonji, hairless and phospholipase A2beta. Trends Biochem Sci *26*, 7-9.

Cortez, C.C., and Jones, P.A. (2008). Chromatin, cancer and drug therapies. Mutat Res *647*, 44-51.

Costa, F.F. (2007). Non-coding RNAs: lost in translation? Gene *386*, 1-10.

Cotto, M., Cabanillas, F., Tirado, M., Garcia, M.V., and Pacheco, E. (2010). Epigenetic therapy of lymphoma using histone deacetylase inhibitors. Clin Transl Oncol *12*, 401-409.

Cremer, T., and Cremer, C. (2001). Chromosome territories, nuclear architecture and gene regulation in mammalian cells. Nat Rev Genet *2*, 292-301.

Cremer, T., and Cremer, M. (2010). Chromosome territories. Cold Spring Harb Perspect Biol *2*, a003889.

Cress, W.D., and Seto, E. (2000). Histone deacetylases, transcriptional control, and cancer. J Cell Physiol *184*, 1-16.

Dalla-Favera, R., Bregni, M., Erikson, J., Patterson, D., Gallo, R.C., and Croce, C.M. (1982). Human c-myc onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. Proc Natl Acad Sci U S A *79*, 7824-7827.

Dasmahapatra, G., Lembersky, D., Son, M.P., Attkisson, E., Dent, P., Fisher, R.I., Friedberg, J.W., and Grant, S. (2011). Carfilzomib interacts synergistically with histone deacetylase inhibitors in mantle cell lymphoma cells in vitro and in vivo. Mol Cancer Ther *10*, 1686-1697.

Dawson, M.A., and Kouzarides, T. (2012). Cancer epigenetics: from mechanism to therapy. Cell *150*, 12-27.

de Boer, C.J., Loyson, S., Kluin, P.M., Kluin-Nelemans, H.C., Schuuring, E., and van Krieken, J.H. (1993). Multiple breakpoints within the BCL-1 locus in B-cell lymphoma: rearrangements of the cyclin D1 gene. Cancer Res *53*, 4148-4152.

De Smet, C., De Backer, O., Faraoni, I., Lurquin, C., Brasseur, F., and Boon, T. (1996). The activation of human gene MAGE-1 in tumor cells is correlated with genome-wide demethylation. Proc Natl Acad Sci U S A *93*, 7149-7153.

De Souza, C., and Chatterji, B.P. (2015). HDAC Inhibitors as Novel Anti-Cancer Therapeutics. Recent Pat Anticancer Drug Discov *10*, 145-162.

DeAngelo, D.J., Spencer, A., Bhalla, K.N., Prince, H.M., Fischer, T., Kindler, T., Giles, F.J., Scott, J.W., Parker, K., Liu, A.*, et al.* (2013). Phase Ia/II, two-arm, open-label, dose-escalation study of oral panobinostat administered via two dosing schedules in patients with advanced hematologic malignancies. Leukemia *27*, 1628-1636.

Deng, S., Calin, G.A., Croce, C.M., Coukos, G., and Zhang, L. (2008). Mechanisms of microRNA deregulation in human cancer. Cell Cycle *7*, 2643-2646.

Dickinson, M., Ritchie, D., DeAngelo, D.J., Spencer, A., Ottmann, O.G., Fischer, T., Bhalla, K.N., Liu, A., Parker, K., Scott, J.W.*, et al.* (2009). Preliminary evidence of disease response to the pan deacetylase inhibitor panobinostat (LBH589) in refractory Hodgkin Lymphoma. Br J Haematol *147*, 97-101.

Dieci, G., Fiorino, G., Castelnuovo, M., Teichmann, M., and Pagano, A. (2007). The expanding RNA polymerase III transcriptome. Trends Genet *23*, 614-622.

Dokmanovic, M., Clarke, C., and Marks, P.A. (2007). Histone deacetylase inhibitors: overview and perspectives. Mol Cancer Res *5*, 981-989.

Dong, M., Ning, Z.Q., Xing, P.Y., Xu, J.L., Cao, H.X., Dou, G.F., Meng, Z.Y., Shi, Y.K., Lu, X.P., and Feng, F.Y. (2012). Phase I study of chidamide (CS055/HBI-8000), a new histone deacetylase inhibitor, in patients with advanced solid tumors and lymphomas. Cancer Chemother Pharmacol *69*, 1413-1422.

Dreyling, M.H., Bullinger, L., Ott, G., Stilgenbauer, S., Muller-Hermelink, H.K., Bentz, M., Hiddemann, W., and Dohner, H. (1997). Alterations of the cyclin D1/p16-pRB pathway in mantle cell lymphoma. Cancer Res *57*, 4608-4614.

Duan, H., Heckman, C.A., and Boxer, L.M. (2005). Histone deacetylase inhibitors down-regulate bcl-2 expression and induce apoptosis in t(14;18) lymphomas. Mol Cell Biol *25*, 1608-1619.

Duvic, M., Dummer, R., Becker, J.C., Poulalhon, N., Ortiz Romero, P., Grazia Bernengo, M., Lebbe, C., Assaf, C., Squier, M., Williams, D.*, et al.* (2013). Panobinostat activity in both bexarotene-exposed and -naive patients with refractory cutaneous T-cell lymphoma: results of a phase II trial. Eur J Cancer *49*, 386-394.

Eden, A., Gaudet, F., Waghmare, A., and Jaenisch, R. (2003). Chromosomal instability and tumors promoted by DNA hypomethylation. Science *300*, 455.

Edmunds, J.W., Mahadevan, L.C., and Clayton, A.L. (2008). Dynamic histone H3 methylation during gene induction: HYPB/Setd2 mediates all H3K36 trimethylation. EMBO J *27*, 406-420.

Ehrlich, M., Buchanan, K.L., Tsien, F., Jiang, G., Sun, B., Uicker, W., Weemaes, C.M., Smeets, D., Sperling, K., Belohradsky, B.H.*, et al.* (2001). DNA methyltransferase 3B mutations linked to the ICF syndrome cause dysregulation of lymphogenesis genes. Hum Mol Genet *10*, 2917-2931.

El-Khoury, V., Pierson, S., Szwarcbart, E., Brons, N.H., Roland, O., Cherrier-De Wilde, S., Plawny, L., Van Dyck, E., and Berchem, G. (2014). Disruption of autophagy by the histone deacetylase inhibitor MGCD0103 and its therapeutic implication in B-cell chronic lymphocytic leukemia. Leukemia *28*, 1636-1646.

Eot-Houllier, G., Fulcrand, G., Magnaghi-Jaulin, L., and Jaulin, C. (2009). Histone deacetylase inhibitors and genomic instability. Cancer Lett *274*, 169-176.

Ernst, T., Chase, A.J., Score, J., Hidalgo-Curtis, C.E., Bryant, C., Jones, A.V., Waghorn, K., Zoi, K., Ross, F.M., Reiter, A.*, et al.* (2010). Inactivating mutations of the histone methyltransferase gene EZH2 in myeloid disorders. Nat Genet *42*, 722-726.

Esteller, M. (2011). Epigenetic changes in cancer. F1000 Biol Rep *3*, 9.

Ewen, M.E., and Lamb, J. (2004). The activities of cyclin D1 that drive tumorigenesis. Trends Mol Med *10*, 158-162.

Farkash-Amar, S., and Simon, I. (2010). Genome-wide analysis of the replication program in mammals. Chromosome Res *18*, 115-125.

Feinberg, A.P., and Tycko, B. (2004). The history of cancer epigenetics. Nat Rev Cancer *4*, 143-153.

Feinberg, A.P., and Vogelstein, B. (1983). Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature *301*, 89-92.

Fernandez, V., Hartmann, E., Ott, G., Campo, E., and Rosenwald, A. (2005). Pathogenesis of mantle-cell lymphoma: all oncogenic roads lead to dysregulation of cell cycle and DNA damage response pathways. J Clin Oncol *23*, 6364-6369.

Fiancette, R., Amin, R., Truffinet, V., Vincent-Fabert, C., Cogne, N., Cogne, M., and Denizot, Y. (2010). A myeloma translocation-like model associating CCND1 with the immunoglobulin heavy-chain locus 3' enhancers does not promote by itself B-cell malignancies. Leuk Res *34*, 1043-1051.

Filion, G.J., van Bemmel, J.G., Braunschweig, U., Talhout, W., Kind, J., Ward, L.D., Brugman, W., de Castro, I.J., Kerkhoven, R.M., Bussemaker, H.J.*, et al.* (2010). Systematic protein location mapping reveals five principal chromatin types in Drosophila cells. Cell *143*, 212-224.

Filipowicz, W., Bhattacharyya, S.N., and Sonenberg, N. (2008). Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet *9*, 102-114.

Filippakopoulos, P., Picaud, S., Mangos, M., Keates, T., Lambert, J.P., Barsyte-Lovejoy, D., Felletar, I., Volkmer, R., Muller, S., Pawson, T.*, et al.* (2012). Histone recognition and large-scale structural analysis of the human bromodomain family. Cell *149*, 214-231.

Fillingham, J., Keogh, M.C., and Krogan, N.J. (2006). GammaH2AX and its role in DNA double-strand break repair. Biochem Cell Biol *84*, 568-577.

Fischle, W., Tseng, B.S., Dormann, H.L., Ueberheide, B.M., Garcia, B.A., Shabanowitz, J., Hunt, D.F., Funabiki, H., and Allis, C.D. (2005). Regulation of HP1-chromatin binding by histone H3 methylation and phosphorylation. Nature *438*, 1116-1122.

Fisher, R.I. (2005). Mantle cell lymphoma: at last, some hope for successful innovative treatment strategies. J Clin Oncol *23*, 657-658.

Fisher, R.I., Dahlberg, S., Nathwani, B.N., Banks, P.M., Miller, T.P., and Grogan, T.M. (1995). A clinical analysis of two indolent lymphoma entities: mantle cell lymphoma and marginal zone lymphoma (including the mucosa-associated lymphoid tissue and monocytoid B-cell subcategories): a Southwest Oncology Group study. Blood *85*, 1075-1082.

Foss, F., Advani, R., Duvic, M., Hymes, K.B., Intragumtornchai, T., Lekhakula, A., Shpilberg, O., Lerner, A., Belt, R.J., Jacobsen, E.D.*, et al.* (2015). A Phase II trial of Belinostat (PXD101) in patients with relapsed or refractory peripheral or cutaneous T-cell lymphoma. Br J Haematol *168*, 811-819.

Fraga, M.F., Ballestar, E., Villar-Garea, A., Boix-Chornet, M., Espada, J., Schotta, G., Bonaldi, T., Haydon, C., Ropero, S., Petrie, K.*, et al.* (2005). Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. Nat Genet *37*, 391-400.

Frank, B., Bermejo, J.L., Hemminki, K., Sutter, C., Wappenschmidt, B., Meindl, A., Kiechle-Bahat, M., Bugert, P., Schmutzler, R.K., Bartram, C.R.*, et al.* (2007). Copy number variant in the candidate tumor suppressor gene MTUS1 and familial breast cancer risk. Carcinogenesis *28*, 1442-1445.

Ganesan, P., Raina, V., and Kumar, R. (2009). A phase II pilot study of valproic acid in relapsed/refractory chronic lymphocytic leukemia. J Clin Oncol *27:15s*, (suppl; abstr 7081).

Garcia-Manero, G., Assouline, S., Cortes, J., Estrov, Z., Kantarjian, H., Yang, H., Newsome, W.M., Miller, W.H., Jr., Rousseau, C., Kalita, A.*, et al.* (2008). Phase 1 study of the oral isotype specific histone deacetylase inhibitor MGCD0103 in leukemia. Blood *112*, 981-989.

Gautsch, J.W., and Wilson, M.C. (1983). Delayed de novo methylation in teratocarcinoma suggests additional tissue-specific mechanisms for controlling gene expression. Nature *301*, 32-37.

Gimsing, P. (2009). Belinostat: a new broad acting antineoplastic histone deacetylase inhibitor. Expert Opin Investig Drugs *18*, 501-508.

Gingras, A.C., Raught, B., and Sonenberg, N. (2001). Regulation of translation initiation by FRAP/mTOR. Genes Dev *15*, 807-826.

Gladden, A.B., and Diehl, J.A. (2003). The cyclin D1-dependent kinase associates with the pre-replication complex and modulates RB.MCM7 binding. J Biol Chem *278*, 9754-9760.

Glozak, M.A., and Seto, E. (2009). Acetylation/deacetylation modulates the stability of DNA replication licensing factor Cdt1. J Biol Chem *284*, 11446-11453.

Goelz, S.E., Vogelstein, B., Hamilton, S.R., and Feinberg, A.P. (1985). Hypomethylation of DNA from benign and malignant human colon neoplasms. Science *228*, 187-190.

Goffin, J., and Eisenhauer, E. (2002). DNA methyltransferase inhibitors-state of the art. Ann Oncol *13*, 1699-1716.

Goldman, M.A. (1988). The chromatin domain as a unit of gene regulation. Bio Essays *9*, 50-55.

Gong, K., Xie, J., Yi, H., and Li, W. (2012). CS055 (Chidamide/HBI-8000), a novel histone deacetylase inhibitor, induces G1 arrest, ROS-dependent apoptosis and differentiation in human leukaemia cells. Biochem J *443*, 735-746.

Gonzalez, E., Kulkarni, H., Bolivar, H., Mangano, A., Sanchez, R., Catano, G., Nibbs, R.J., Freedman, B.I., Quinones, M.P., Bamshad, M.J.*, et al.* (2005). The influence of CCL3L1 gene-containing segmental duplications on HIV-1/AIDS susceptibility. Science *307*, 1434-1440.

Grafodatskaya, D., Chung, B., Szatmari, P., and Weksberg, R. (2010). Autism spectrum disorders and epigenetics. J Am Acad Child Adolesc Psychiatry *49*, 794-809.

Grant, C., Rahman, F., Piekarz, R., Peer, C., Frye, R., Robey, R.W., Gardner, E.R., Figg, W.D., and Bates, S.E. (2010). Romidepsin: a new therapy for cutaneous T-cell lymphoma and a potential therapy for solid tumors. Expert Rev Anticancer Ther *10*, 997-1008.

Grant, S., Easley, C., and Kirkpatrick, P. (2007). Vorinostat. Nat Rev Drug Discov *6*, 21-22.

Gray, S.G., Qian, C.N., Furge, K., Guo, X., and Teh, B.T. (2004). Microarray profiling of the effects of histone deacetylase inhibitors on gene expression in cancer cell lines. Int J Oncol *24*, 773-795.

Greger, V., Passarge, E., Hopping, W., Messmer, E., and Horsthemke, B. (1989). Epigenetic changes may contribute to the formation and spontaneous regression of retinoblastoma. Hum Genet *83*, 155-158.

Greiner, T.C., Dasgupta, C., Ho, V.V., Weisenburger, D.D., Smith, L.M., Lynch, J.C., Vose, J.M., Fu, K., Armitage, J.O., Braziel, R.M.*, et al.* (2006). Mutation and genomic deletion status of ataxia telangiectasia mutated (ATM) and p53 confer specific gene expression profiles in mantle cell lymphoma. Proc Natl Acad Sci U S A *103*, 2352-2357.

Grignani, F., De Matteis, S., Nervi, C., Tomassoni, L., Gelmetti, V., Cioce, M., Fanelli, M., Ruthardt, M., Ferrara, F.F., Zamir, I.*, et al.* (1998a). Fusion proteins of the retinoic acid receptor-alpha recruit histone deacetylase in promyelocytic leukaemia. Nature *391*, 815-818.

Grignani, F., De Matteis, S., Nervi, C., Tomassoni, L., Gelmetti, V., Cioce, M., Fanelli, M., Ruthardt, M., Ferrara, F.F., Zamir, I.*, et al.* (1998b). Fusion proteins of the retinoic acid receptor-alpha recruit histone deacetylase in promyelocytic leukaemia. Nature *391*, 815-818.

Gruhn, B., Naumann, T., Gruner, D., Walther, M., Wittig, S., Becker, S., Beck, J.F., and Sonnemann, J. (2013). The expression of histone deacetylase 4 is associated with prednisone poor-response in childhood acute lymphoblastic leukemia. Leuk Res *37*, 1200-1207.

Gu, W., and Roeder, R.G. (1997). Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. Cell *90*, 595-606.

Guttman, M., Amit, I., Garber, M., French, C., Lin, M.F., Feldser, D., Huarte, M., Zuk, O., Carey, B.W., Cassady, J.P.*, et al.* (2009). Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. Nature *458*, 223-227.

Guttman, M., Donaghey, J., Carey, B.W., Garber, M., Grenier, J.K., Munson, G., Young, G., Lucas, A.B., Ach, R., Bruhn, L.*, et al.* (2011). lincRNAs act in the circuitry controlling pluripotency and differentiation. Nature *477*, 295-300.

Hadorn, E. (1961). Developmental Genetics and Lethal Factors. Methuen, London.

Haggarty, S.J., Koeller, K.M., Wong, J.C., Grozinger, C.M., and Schreiber, S.L. (2003). Domain-selective small-molecule inhibitor of histone deacetylase 6 (HDAC6)-mediated tubulin deacetylation. Proc Natl Acad Sci U S A *100*, 4389-4394.

Halkidou, K., Gaughan, L., Cook, S., Leung, H.Y., Neal, D.E., and Robson, C.N. (2004). Upregulation and nuclear recruitment of HDAC1 in hormone refractory prostate cancer. Prostate *59*, 177-189.

Hall, I.M., Noma, K., and Grewal, S.I. (2003). RNA interference machinery regulates chromosome dynamics during mitosis and meiosis in fission yeast. Proc Natl Acad Sci U S A *100*, 193-198.

Halsall, J., Gupta, V., O'Neill, L.P., Turner, B.M., and Nightingale, K.P. (2012). Genes are often sheltered from the global histone hyperacetylation induced by HDAC inhibitors. PLoS One *7*, e33453.

Han, Y., Amin, H.M., Frantz, C., Franko, B., Lee, J., Lin, Q., and Lai, R. (2006). Restoration of shp1 expression by 5-AZA-2'-deoxycytidine is associated with downregulation of JAK3/STAT3 signaling in ALK-positive anaplastic large cell lymphoma. Leukemia *20*, 1602-1609.

Harewood, L., Schutz, F., Boyle, S., Perry, P., Delorenzi, M., Bickmore, W.A., and Reymond, A. (2010). The effect of translocation-induced nuclear reorganization on gene expression. Genome Res *20*, 554-564.

Hassa, P.O., Haenni, S.S., Elser, M., and Hottiger, M.O. (2006). Nuclear ADP-ribosylation reactions in mammalian cells: where are we today and where are we going? Microbiol Mol Biol Rev *70*, 789-829.

Hassler, M.R., Klisaroska, A., Kollmann, K., Steiner, I., Bilban, M., Schiefer, A.I., Sexl, V., and Egger, G. (2012). Antineoplastic activity of the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine in anaplastic large cell lymphoma. Biochimie *94*, 2297-2307.

Hassler, M.R., Schiefer, A.I., and Egger, G. (2013). Combating the epigenome: epigenetic drugs against non-Hodgkin's lymphoma. Epigenomics *5*, 397-415.

Hatzimichael, E., and Crook, T. (2013). Cancer Epigenetics: New Therapies and New Challenges. Journal of Drug Delivery *2013*, 9.

Hauser, C., Schuettengruber, B., Bartl, S., Lagger, G., and Seiser, C. (2002). Activation of the mouse histone deacetylase 1 gene by cooperative histone phosphorylation and acetylation. Mol Cell Biol *22*, 7820-7830.

Heideman, M.R., Wilting, R.H., Yanover, E., Velds, A., de Jong, J., Kerkhoven, R.M., Jacobs, H., Wessels, L.F., and Dannenberg, J.H. (2013). Dosage-dependent tumor suppression by histone deacetylases 1 and 2 through regulation of c-Myc collaborating genes and p53 function. Blood *121*, 2038-2050.

Heider, U., Kaiser, M., Sterz, J., Zavrski, I., Jakob, C., Fleissner, C., Eucker, J., Possinger, K., and Sezer, O. (2006). Histone deacetylase inhibitors reduce VEGF production and induce growth suppression and apoptosis in human mantle cell lymphoma. Eur J Haematol *76*, 42-50.

Heltweg, B., Gatbonton, T., Schuler, A.D., Posakony, J., Li, H., Goehle, S., Kollipara, R., Depinho, R.A., Gu, Y., Simon, J.A.*, et al.* (2006). Antitumor activity of a small-molecule inhibitor of human silent information regulator 2 enzymes. Cancer Res *66*, 4368-4377.

Hendrich, B., and Bird, A. (1998). Identification and characterization of a family of mammalian methyl-CpG binding proteins. Mol Cell Biol *18*, 6538-6547.

Heng, H.H., Krawetz, S.A., Lu, W., Bremer, S., Liu, G., and Ye, C.J. (2001). Re-defining the chromatin loop domain. Cytogenet Cell Genet *93*, 155-161.

Holliday, R., and Pugh, J.E. (1975). DNA modification mechanisms and gene activity during development. Science *187*, 226-232.

Houwing, S., Kamminga, L.M., Berezikov, E., Cronembold, D., Girard, A., van den Elst, H., Filippov, D.V., Blaser, H., Raz, E., Moens, C.B.*, et al.* (2007). A role for Piwi and piRNAs in germ cell maintenance and transposon silencing in Zebrafish. Cell *129*, 69-82.

Howard, G., Eiges, R., Gaudet, F., Jaenisch, R., and Eden, A. (2008). Activation and transposition of endogenous retroviral elements in hypomethylation induced tumors in mice. Oncogene *27*, 404-408.

Iguchi-Ariga, S.M., and Schaffner, W. (1989). CpG methylation of the cAMP-responsive enhancer/promoter sequence TGACGTCA abolishes specific factor binding as well as transcriptional activation. Genes Dev *3*, 612-619.

Insinga, A., Monestiroli, S., Ronzoni, S., Gelmetti, V., Marchesi, F., Viale, A., Altucci, L., Nervi, C., Minucci, S., and Pelicci, P.G. (2005). Inhibitors of histone deacetylases induce tumor-selective apoptosis through activation of the death receptor pathway. Nat Med *11*, 71-76.

Ishii, Y., Pirkmaier, A., Alvarez, J.V., Frank, D.A., Keselman, I., Logothetis, D., Mandeli, J., O'Connell, M.J., Waxman, S., and Germain, D. (2006). Cyclin D1 overexpression and response to bortezomib treatment in a breast cancer model. J Natl Cancer Inst *98*, 1238-1247.

Issa, J.P. (2000). CpG-island methylation in aging and cancer. Curr Top Microbiol Immunol *249*, 101-118.

Issa, J.P., Garcia-Manero, G., Giles, F.J., Mannari, R., Thomas, D., Faderl, S., Bayar, E., Lyons, J., Rosenfeld, C.S., Cortes, J.*, et al.* (2004). Phase 1 study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2'-deoxycytidine (decitabine) in hematopoietic malignancies. Blood *103*, 1635-1640.

Jackson-Grusby, L., Beard, C., Possemato, R., Tudor, M., Fambrough, D., Csankovszki, G., Dausman, J., Lee, P., Wilson, C., Lander, E.*, et al.* (2001). Loss of genomic methylation causes p53-dependent apoptosis and epigenetic deregulation. Nat Genet *27*, 31-39.

Jaenisch, R., Harbers, K., Jahner, D., Stewart, C., and Stuhlmann, H. (1982). DNA methylation, retroviruses, and embryogenesis. J Cell Biochem *20*, 331-336.

Jaffe, E., Harris, N., Stein, H., and al., e., eds. (2001). Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues (Lyon, France, IARC Press).

Jenuwein, T., and Allis, C.D. (2001). Translating the histone code. Science *293*, 1074-1080.

Jin, Y., Cao, Q., Chen, C., Du, X., Jin, B., and Pan, J. (2015). Tenovin-6-mediated inhibition of SIRT1/2 induces apoptosis in acute lymphoblastic leukemia (ALL) cells and eliminates ALL stem/progenitor cells. BMC Cancer *15*, 226.

Jones, P.A. (2008). Moving AHEAD with an international human epigenome project. Nature *454*, 711-715.

Jones, P.A., and Baylin, S.B. (2002). The fundamental role of epigenetic events in cancer. Nat Rev Genet *3*, 415-428.

Jones, P.A., and Laird, P.W. (1999). Cancer epigenetics comes of age. Nat Genet *21*, 163-167.

Jones, P.L., Veenstra, G.J., Wade, P.A., Vermaak, D., Kass, S.U., Landsberger, N., Strouboulis, J., and Wolffe, A.P. (1998). Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat Genet *19*, 187-191.

Kantarjian, H.M., O'Brien, S., Cortes, J., Giles, F.J., Faderl, S., Issa, J.P., Garcia-Manero, G., Rios, M.B., Shan, J., Andreeff, M.*, et al.* (2003). Results of decitabine (5-aza-2'deoxycytidine) therapy in 130 patients with chronic myelogenous leukemia. Cancer *98*, 522-528.

Kaplan, L.D., Deitcher, S.R., Silverman, J.A., and Morgan, G. (2014). Phase II study of vincristine sulfate liposome injection (Marqibo) and rituximab for patients with relapsed and

refractory diffuse large B-Cell lymphoma or mantle cell lymphoma in need of palliative therapy. Clin Lymphoma Myeloma Leuk *14*, 37-42.

Katayama, S., Tomaru, Y., Kasukawa, T., Waki, K., Nakanishi, M., Nakamura, M., Nishida, H., Yap, C.C., Suzuki, M., Kawai, J.*, et al.* (2005). Antisense transcription in the mammalian transcriptome. Science *309*, 1564-1566.

Kawamata, N., Chen, J., and Koeffler, H.P. (2007). Suberoylanilide hydroxamic acid (SAHA; vorinostat) suppresses translation of cyclin D1 in mantle cell lymphoma cells. Blood *110*, 2667-2673.

Kim, H.J., and Bae, S.C. (2011). Histone deacetylase inhibitors: molecular mechanisms of action and clinical trials as anti-cancer drugs. Am J Transl Res *3*, 166-179.

Kirschbaum, M., Frankel, P., Popplewell, L., Zain, J., Delioukina, M., Pullarkat, V., Matsuoka, D., Pulone, B., Rotter, A.J., Espinoza-Delgado, I.*, et al.* (2011). Phase II study of vorinostat for treatment of relapsed or refractory indolent non-Hodgkin's lymphoma and mantle cell lymphoma. J Clin Oncol *29*, 1198-1203.

Kirsh, O., Seeler, J.S., Pichler, A., Gast, A., Muller, S., Miska, E., Mathieu, M., Harel-Bellan, A., Kouzarides, T., Melchior, F.*, et al.* (2002). The SUMO E3 ligase RanBP2 promotes modification of the HDAC4 deacetylase. EMBO J *21*, 2682-2691.

Klimek, V.M., Fircanis, S., Maslak, P., Guernah, I., Baum, M., Wu, N., Panageas, K., Wright, J.J., Pandolfi, P.P., and Nimer, S.D. (2008). Tolerability, pharmacodynamics, and pharmacokinetics studies of depsipeptide (romidepsin) in patients with acute myelogenous leukemia or advanced myelodysplastic syndromes. Clin Cancer Res *14*, 826-832.

Klose, R.J., and Zhang, Y. (2007). Regulation of histone methylation by demethylimination and demethylation. Nat Rev Mol Cell Biol *8*, 307-318.

Kluiver, J., Kroesen, B.J., Poppema, S., and van den Berg, A. (2006). The role of microRNAs in normal hematopoiesis and hematopoietic malignancies. Leukemia *20*, 1931-1936.

Kn, H., Bassal, S., Tikellis, C., and El-Osta, A. (2004). Expression analysis of the epigenetic methyltransferases and methyl-CpG binding protein families in the normal B-cell and B-cell chronic lymphocytic leukemia (CLL). Cancer Biol Ther *3*, 989-994.

Knight, S.J., Regan, R., Nicod, A., Horsley, S.W., Kearney, L., Homfray, T., Winter, R.M., Bolton, P., and Flint, J. (1999). Subtle chromosomal rearrangements in children with unexplained mental retardation. Lancet *354*, 1676-1681.

Koch, C.M., Andrews, R.M., Flicek, P., Dillon, S.C., Karaoz, U., Clelland, G.K., Wilcox, S., Beare, D.M., Fowler, J.C., Couttet, P.*, et al.* (2007). The landscape of histone modifications across 1% of the human genome in five human cell lines. Genome Res *17*, 691-707.

Kodani, M., Igishi, T., Matsumoto, S., Chikumi, H., Shigeoka, Y., Nakanishi, H., Morita, M., Yasuda, K., Hitsuda, Y., and Shimizu, E. (2005). Suppression of phosphatidylinositol 3-kinase/Akt signaling pathway is a determinant of the sensitivity to a novel histone deacetylase inhibitor, FK228, in lung adenocarcinoma cells. Oncol Rep *13*, 477-483.

Koprivnikar, J.L., and Cheson, B.D. (2012). Bortezomib: a proteasome inhibitor with an evolving role in select subtypes of B-cell non-Hodgkin's lymphoma. Future Oncol *8*, 359-371.

Korsmeyer, S.J. (1992). Chromosomal translocations in lymphoid malignancies reveal novel proto-oncogenes. Annu Rev Immunol *10*, 785-807.

Kouzarides, T. (2002). Histone methylation in transcriptional control. Curr Opin Genet Dev *12*, 198-209.

Kouzarides, T. (2007). Chromatin modifications and their function. Cell *128*, 693-705.

Krishnamoorthy, T., Chen, X., Govin, J., Cheung, W.L., Dorsey, J., Schindler, K., Winter, E., Allis, C.D., Guacci, V., Khochbin, S.*, et al.* (2006). Phosphorylation of histone H4 Ser1 regulates sporulation in yeast and is conserved in fly and mouse spermatogenesis. Genes Dev *20*, 2580-2592.

Kristensen, L.S., Nielsen, H.M., and Hansen, L.L. (2009). Epigenetics and cancer treatment. Eur J Pharmacol *625*, 131-142.

Lacoste, N., Utley, R.T., Hunter, J.M., Poirier, G.G., and Cote, J. (2002). Disruptor of telomeric silencing-1 is a chromatin-specific histone H3 methyltransferase. J Biol Chem *277*, 30421-30424.

Laird, P.W. (2003). The power and the promise of DNA methylation markers. Nat Rev Cancer *3*, 253-266.

Lawson, G.M., Knoll, B.J., March, C.J., Woo, S.L., Tsai, M.J., and O'Malley, B.W. (1982). Definition of 5' and 3' structural boundaries of the chromatin domain containing the ovalbumin multigene family. J Biol Chem *257*, 1501-1507.

Lee, H.J., and Kim, K.W. (2012). Suppression of HIF-1alpha by Valproic Acid Sustains Self-Renewal of Mouse Embryonic Stem Cells under Hypoxia In Vitro. Biomol Ther (Seoul) *20*, 280-285.

Lee, J.H., Choy, M.L., Ngo, L., Foster, S.S., and Marks, P.A. (2010). Histone deacetylase inhibitor induces DNA damage, which normal but not transformed cells can repair. Proc Natl Acad Sci U S A *107*, 14639-14644.

Lee, J.T. (2009). Lessons from X-chromosome inactivation: long ncRNA as guides and tethers to the epigenome. Genes Dev *23*, 1831-1842.

Lee, S.H., Yoo, C., Im, S., Jung, J.H., Choi, H.J., and Yoo, J. (2014). Expression of histone deacetylases in diffuse large B-cell lymphoma and its clinical significance. Int J Med Sci *11*, 994-1000.

Legube, G., Linares, L.K., Lemercier, C., Scheffner, M., Khochbin, S., and Trouche, D. (2002). Tip60 is targeted to proteasome-mediated degradation by Mdm2 and accumulates after UV irradiation. EMBO J *21*, 1704-1712.

Legube, G., and Trouche, D. (2003). Regulating histone acetyltransferases and deacetylases. EMBO Rep *4*, 944-947.

Lesne, A., Foray, N., Cathala, G., Forne, T., Wong, H., and Victor, J.M. (2015). Chromatin fiber allostery and the epigenetic code. J Phys Condens Matter *27*, 064114.

Ley, T.J., Ding, L., Walter, M.J., McLellan, M.D., Lamprecht, T., Larson, D.E., Kandoth, C., Payton, J.E., Baty, J., Welch, J.*, et al.* (2010). DNMT3A mutations in acute myeloid leukemia. N Engl J Med *363*, 2424-2433.

Li, E. (2002). Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genet *3*, 662-673.

Li, E., and Zhang, Y. (2014). DNA methylation in mammals. Cold Spring Harb Perspect Biol *6*, a019133.

Li, L., Wang, L., Wang, Z., Ho, Y., McDonald, T., Holyoake, T.L., Chen, W., and Bhatia, R. (2012). Activation of p53 by SIRT1 inhibition enhances elimination of CML leukemia stem cells in combination with imatinib. Cancer Cell *21*, 266-281.

Li, L.C., Okino, S.T., Zhao, H., Pookot, D., Place, R.F., Urakami, S., Enokida, H., and Dahiya, R. (2006). Small dsRNAs induce transcriptional activation in human cells. Proc Natl Acad Sci U S A *103*, 17337-17342.

Lin, R.J., and Evans, R.M. (2000). Acquisition of oncogenic potential by RAR chimeras in acute promyelocytic leukemia through formation of homodimers. Mol Cell *5*, 821-830.

Liu, H., Wang, J., and Epner, E.M. (2004). Cyclin D1 activation in B-cell malignancy: association with changes in histone acetylation, DNA methylation, and RNA polymerase II binding to both promoter and distal sequences. Blood *104*, 2505-2513.

Liu, Z., Xie, Z., Jones, W., Pavlovicz, R.E., Liu, S., Yu, J., Li, P.K., Lin, J., Fuchs, J.R., Marcucci, G.*, et al.* (2009). Curcumin is a potent DNA hypomethylation agent. Bioorg Med Chem Lett *19*, 706-709.

Lock, L.F., Takagi, N., and Martin, G.R. (1987). Methylation of the Hprt gene on the inactive X occurs after chromosome inactivation. Cell *48*, 39-46.

Lopez-Atalaya, J.P., Ito, S., Valor, L.M., Benito, E., and Barco, A. (2013). Genomic targets, and histone acetylation and gene expression profiling of neural HDAC inhibition. Nucleic Acids Res *41*, 8072-8084.

Lu, J., Getz, G., Miska, E.A., Alvarez-Saavedra, E., Lamb, J., Peck, D., Sweet-Cordero, A., Ebert, B.L., Mak, R.H., Ferrando, A.A.*, et al.* (2005). MicroRNA expression profiles classify human cancers. Nature *435*, 834-838.

Luger, K., Mader, A.W., Richmond, R.K., Sargent, D.F., and Richmond, T.J. (1997). Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature *389*, 251-260.

Lupski, J.R. (1998). Genomic disorders: structural features of the genome can lead to DNA rearrangements and human disease traits. Trends Genet *14*, 417-422.

Maier, S., and Olek, A. (2002). Diabetes: a candidate disease for efficient DNA methylation profiling. J Nutr *132*, 2440S-2443S.

Markozashvili, D., Ribrag, V., and Vassetzky, Y.S. (2015). Histone deacetylase inhibitors and epigenetic regulation in lymphoid malignancies. Invest New Drugs.

Martin, C., and Zhang, Y. (2005). The diverse functions of histone lysine methylation. Nat Rev Mol Cell Biol *6*, 838-849.

Martinez-Garcia, E., Popovic, R., Min, D.J., Sweet, S.M., Thomas, P.M., Zamdborg, L., Heffner, A., Will, C., Lamy, L., Staudt, L.M.*, et al.* (2011). The MMSET histone methyl transferase switches global histone methylation and alters gene expression in t(4;14) multiple myeloma cells. Blood *117*, 211-220.

Matsuyama, A., Shimazu, T., Sumida, Y., Saito, A., Yoshimatsu, Y., Seigneurin-Berny, D., Osada, H., Komatsu, Y., Nishino, N., Khochbin, S.*, et al.* (2002). In vivo destabilization of dynamic microtubules by HDAC6-mediated deacetylation. EMBO J *21*, 6820-6831.

Mattick, J.S. (2001). Non-coding RNAs: the architects of eukaryotic complexity. EMBO Rep *2*, 986-991.

Meissner, A., Mikkelsen, T.S., Gu, H., Wernig, M., Hanna, J., Sivachenko, A., Zhang, X., Bernstein, B.E., Nusbaum, C., Jaffe, D.B.*, et al.* (2008). Genome-scale DNA methylation maps of pluripotent and differentiated cells. Nature *454*, 766-770.

Mercer, T.R., and Mattick, J.S. (2013). Structure and function of long noncoding RNAs in epigenetic regulation. Nat Struct Mol Biol *20*, 300-307.

Methot, J.L., Chakravarty, P.K., Chenard, M., Close, J., Cruz, J.C., Dahlberg, W.K., Fleming, J., Hamblett, C.L., Hamill, J.E., Harrington, P.*, et al.* (2008). Exploration of the internal cavity of histone deacetylase (HDAC) with selective HDAC1/HDAC2 inhibitors (SHI-1:2). Bioorg Med Chem Lett *18*, 973-978.

Mikkelsen, T.S., Ku, M., Jaffe, D.B., Issac, B., Lieberman, E., Giannoukos, G., Alvarez, P., Brockman, W., Kim, T.K., Koche, R.P.*, et al.* (2007). Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. Nature *448*, 553-560.

Milagro, F.I., Campion, J., Cordero, P., Goyenechea, E., Gomez-Uriz, A.M., Abete, I., Zulet, M.A., and Martinez, J.A. (2011). A dual epigenomic approach for the search of obesity biomarkers: DNA methylation in relation to diet-induced weight loss. FASEB J *25*, 1378-1389.

Mills, A.A. (2010). Throwing the cancer switch: reciprocal roles of polycomb and trithorax proteins. Nat Rev Cancer *10*, 669-682.

Min, S.K., Koh, Y.H., Park, Y., Kim, H.J., Seo, J., Park, H.R., Cho, S.J., and Kim, I.S. (2012). Expression of HAT1 and HDAC1, 2, 3 in Diffuse Large B-Cell Lymphomas, Peripheral T-Cell Lymphomas, and NK/T-Cell Lymphomas. Korean J Pathol *46*, 142-150.

Minucci, S., Maccarana, M., Cioce, M., De Luca, P., Gelmetti, V., Segalla, S., Di Croce, L., Giavara, S., Matteucci, C., Gobbi, A.*, et al.* (2000). Oligomerization of RAR and AML1 transcription factors as a novel mechanism of oncogenic activation. Mol Cell *5*, 811-820.

Mitelman, F., Johansson, B., and Mertens, F. (2004). Fusion genes and rearranged genes as a linear function of chromosome aberrations in cancer. Nat Genet *36*, 331-334.

Mitelman, F., Johansson, B., and Mertens, F. (2007). The impact of translocations and gene fusions on cancer causation. Nat Rev Cancer *7*, 233-245.

Morin, R.D., Mendez-Lago, M., Mungall, A.J., Goya, R., Mungall, K.L., Corbett, R.D., Johnson, N.A., Severson, T.M., Chiu, R., Field, M.*, et al.* (2011). Frequent mutation of histone-modifying genes in non-Hodgkin lymphoma. Nature *476*, 298-303.

Morris, K.V., Chan, S.W., Jacobsen, S.E., and Looney, D.J. (2004). Small interfering RNA-induced transcriptional gene silencing in human cells. Science *305*, 1289-1292.

Morschhauser, F., Terriou, L., Coiffier, B., Bachy, E., Varga, A., Kloos, I., Lelievre, H., Sarry, A.L., Depil, S., and Ribrag, V. (2015). Phase 1 study of the oral histone deacetylase inhibitor abexinostat in patients with Hodgkin lymphoma, non-Hodgkin lymphoma, or chronic lymphocytic leukaemia. Invest New Drugs *33*, 423-431.

Nagy, Z., and Tora, L. (2007). Distinct GCN5/PCAF-containing complexes function as co-activators and are involved in transcription factor and global histone acetylation. Oncogene *26*, 5341-5357.

Nakajima, H., Kim, Y.B., Terano, H., Yoshida, M., and Horinouchi, S. (1998). FR901228, a potent antitumor antibiotic, is a novel histone deacetylase inhibitor. Exp Cell Res *241*, 126-133.

Nambiar, M., Kari, V., and Raghavan, S.C. (2008). Chromosomal translocations in cancer. Biochim Biophys Acta *1786*, 139-152.

Nathan, D., Ingvarsdottir, K., Sterner, D.E., Bylebyl, G.R., Dokmanovic, M., Dorsey, J.A., Whelan, K.A., Krsmanovic, M., Lane, W.S., Meluh, P.B.*, et al.* (2006). Histone sumoylation is a negative regulator in Saccharomyces cerevisiae and shows dynamic interplay with positive-acting histone modifications. Genes Dev *20*, 966-976.

Nephew, K.P., and Huang, T.H. (2003). Epigenetic gene silencing in cancer initiation and progression. Cancer Lett *190*, 125-133.

Neuwald, A.F., and Landsman, D. (1997). GCN5-related histone N-acetyltransferases belong to a diverse superfamily that includes the yeast SPT10 protein. Trends Biochem Sci *22*, 154-155.

New, M., Olzscha, H., and La Thangue, N.B. (2012). HDAC inhibitor-based therapies: can we interpret the code? Mol Oncol *6*, 637-656.

Ng, H.H., and Bird, A. (1999). DNA methylation and chromatin modification. Curr Opin Genet Dev *9*, 158-163.

Nicol, J.W., Helt, G.A., Blanchard, S.G., Jr., Raja, A., and Loraine, A.E. (2009). The Integrated Genome Browser: free software for distribution and exploration of genome-scale datasets. Bioinformatics *25*, 2730-2731.

Nikoloski, G., Langemeijer, S.M., Kuiper, R.P., Knops, R., Massop, M., Tonnissen, E.R., van der Heijden, A., Scheele, T.N., Vandenberghe, P., de Witte, T.*, et al.* (2010). Somatic mutations of the histone methyltransferase gene EZH2 in myelodysplastic syndromes. Nat Genet *42*, 665-667.

North, J.A., Simon, M., Ferdinand, M.B., Shoffner, M.A., Picking, J.W., Howard, C.J., Mooney, A.M., van Noort, J., Poirier, M.G., and Ottesen, J.J. (2014). Histone H3 phosphorylation near the nucleosome dyad alters chromatin structure. Nucleic Acids Res *42*, 4922-4933.

Nowell, P.C.a.H., D. A. (1960). A minute chromosome in human chronic granulocytic leukemia. Science *132*, 1497.

Nussenzweig, A., and Nussenzweig, M.C. (2010). Origin of chromosomal translocations in lymphoid cancer. Cell *141*, 27-38.

O'Donnell, K.A., and Boeke, J.D. (2007). Mighty Piwis defend the germline against genome intruders. Cell *129*, 37-44.

Ogama, Y., Ouchida, M., Yoshino, T., Ito, S., Takimoto, H., Shiote, Y., Ishimaru, F., Harada, M., Tanimoto, M., and Shimizu, K. (2004). Prevalent hyper-methylation of the CDH13 gene promoter in malignant B cell lymphomas. Int J Oncol *25*, 685-691.

Ogura, M., Ando, K., Suzuki, T., Ishizawa, K., Oh, S.Y., Itoh, K., Yamamoto, K., Au, W.Y., Tien, H.F., Matsuno, Y.*, et al.* (2014). A multicentre phase II study of vorinostat in patients with relapsed or refractory indolent B-cell non-Hodgkin lymphoma and mantle cell lymphoma. Br J Haematol *165*, 768-776.

Ohno, H. (2004). Pathogenetic role of BCL6 translocation in B-cell non-Hodgkin's lymphoma. Histol Histopathol *19*, 637-650.

Pannell, D., Osborne, C.S., Yao, S., Sukonnik, T., Pasceri, P., Karaiskakis, A., Okano, M., Li, E., Lipshitz, H.D., and Ellis, J. (2000). Retrovirus vector silencing is de novo methylase independent and marked by a repressive histone code. EMBO J *19*, 5884-5894.

Parra, M. (2009). Epigenetic events during B lymphocyte development. Epigenetics *4*, 462-468.

Pasqualucci, L., Dominguez-Sola, D., Chiarenza, A., Fabbri, G., Grunn, A., Trifonov, V., Kasper, L.H., Lerach, S., Tang, H., Ma, J.*, et al.* (2011). Inactivating mutations of acetyltransferase genes in B-cell lymphoma. Nature *471*, 189-195.

Pavri, R., Zhu, B., Li, G., Trojer, P., Mandal, S., Shilatifard, A., and Reinberg, D. (2006). Histone H2B monoubiquitination functions cooperatively with FACT to regulate elongation by RNA polymerase II. Cell *125*, 703-717.

Peart, M.J., Smyth, G.K., van Laar, R.K., Bowtell, D.D., Richon, V.M., Marks, P.A., Holloway, A.J., and Johnstone, R.W. (2005). Identification and functional significance of genes regulated by structurally different histone deacetylase inhibitors. Proc Natl Acad Sci U S A *102*, 3697-3702.

Peters, A.H., O'Carroll, D., Scherthan, H., Mechtler, K., Sauer, S., Schofer, C., Weipoltshammer, K., Pagani, M., Lachner, M., Kohlmaier, A.*, et al.* (2001). Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability. Cell *107*, 323-337.

Pham, L.V., Tamayo, A.T., Yoshimura, L.C., Lo, P., and Ford, R.J. (2003). Inhibition of constitutive NF-kappa B activation in mantle cell lymphoma B cells leads to induction of cell cycle arrest and apoptosis. J Immunol *171*, 88-95.

Piekarz, R.L., Frye, R., Turner, M., Wright, J.J., Allen, S.L., Kirschbaum, M.H., Zain, J., Prince, H.M., Leonard, J.P., Geskin, L.J.*, et al.* (2009). Phase II multi-institutional trial of the histone deacetylase inhibitor romidepsin as monotherapy for patients with cutaneous T-cell lymphoma. J Clin Oncol *27*, 5410-5417.

Pili, R., Salumbides, B., Zhao, M., Altiok, S., Qian, D., Zwiebel, J., Carducci, M.A., and Rudek, M.A. (2012). Phase I study of the histone deacetylase inhibitor entinostat in combination with 13-cis retinoic acid in patients with solid tumours. Br J Cancer *106*, 77-84.

Pui, C.H., Relling, M.V., and Downing, J.R. (2004). Acute lymphoblastic leukemia. N Engl J Med *350*, 1535-1548.

Rabl, C. (1885). Uber Zellteilung. In Morpholgisches Jahrbuch, G. C., ed., pp. 214-258.

Rajneesh, R., and Sinha, R. (2014). Hydroxymethylation of DNA: an epigenetic marker. EXCLI Journal *13*, 592-610.

Rambaldi, A., Dellacasa, C.M., Finazzi, G., Carobbio, A., Ferrari, M.L., Guglielmelli, P., Gattoni, E., Salmoiraghi, S., Finazzi, M.C., Di Tollo, S.*, et al.* (2010). A pilot study of the Histone-Deacetylase inhibitor Givinostat in patients with JAK2V617F positive chronic myeloproliferative neoplasms. Br J Haematol *150*, 446-455.

Rando, O.J. (2012). Combinatorial complexity in chromatin structure and function: revisiting the histone code. Curr Opin Genet Dev *22*, 148-155.

Rao, R., Balusu, R., Fiskus, W., Mudunuru, U., Venkannagari, S., Chauhan, L., Smith, J.E., Hembruff, S.L., Ha, K., Atadja, P.*, et al.* (2012). Combination of pan-histone deacetylase inhibitor and autophagy inhibitor exerts superior efficacy against triple-negative human breast cancer cells. Mol Cancer Ther *11*, 973-983.

Razin, S.V., Iarovaia, O.V., Sjakste, N., Sjakste, T., Bagdoniene, L., Rynditch, A.V., Eivazova, E.R., Lipinski, M., and Vassetzky, Y.S. (2007). Chromatin domains and regulation of transcription. J Mol Biol *369*, 597-607.

Razin, S.V., Iarovaia, O.V., and Vassetzky, Y.S. (2014). A requiem to the nuclear matrix: from a controversial concept to 3D organization of the nucleus. Chromosoma *123*, Doi: 10.1007/s00412-00014-00459-00418.

Rege-Cambrin, G., Giugliano, E., Michaux, L., Stul, M., Scaravaglio, P., Serra, A., Saglio, G., and Hagemeijer, A. (2005). Trisomy 11 in myeloid malignancies is associated with internal tandem duplication of both MLL and FLT3 genes. Haematologica *90*, 262-264.

Rhee, I., Jair, K.W., Yen, R.W., Lengauer, C., Herman, J.G., Kinzler, K.W., Vogelstein, B., Baylin, S.B., and Schuebel, K.E. (2000). CpG methylation is maintained in human cancer cells lacking DNMT1. Nature *404*, 1003-1007.

Richman, R., Chicoine, L.G., Collini, M.P., Cook, R.G., and Allis, C.D. (1988). Micronuclei and the cytoplasm of growing Tetrahymena contain a histone acetylase activity which is highly specific for free histone H4. J Cell Biol *106*, 1017-1026.

Richon, V.M., Emiliani, S., Verdin, E., Webb, Y., Breslow, R., Rifkind, R.A., and Marks, P.A. (1998). A class of hybrid polar inducers of transformed cell differentiation inhibits histone deacetylases. Proc Natl Acad Sci U S A *95*, 3003-3007.

Richon, V.M., and O'Brien, J.P. (2002). Histone deacetylase inhibitors: a new class of potential therapeutic agents for cancer treatment. Clin Cancer Res *8*, 662-664.

Riggs, M.G., Whittaker, R.G., Neumann, J.R., and Ingram, V.M. (1977). n-Butyrate causes histone modification in HeLa and Friend erythroleukaemia cells. Nature *268*, 462-464.

Rimokh, R., Berger, F., Delsol, G., Charrin, C., Bertheas, M.F., Ffrench, M., Garoscio, M., Felman, P., Coiffier, B., Bryon, P.A.*, et al.* (1993). Rearrangement and overexpression of the BCL-1/PRAD-1 gene in intermediate lymphocytic lymphomas and in t(11q13)-bearing leukemias. Blood *81*, 3063-3067.

Rivera-Del Valle, N., Gao, S., Miller, C.P., Fulbright, J., Gonzales, C., Sirisawad, M., Steggerda, S., Wheler, J., Balasubramanian, S., and Chandra, J. (2010). PCI-24781, a Novel Hydroxamic Acid HDAC Inhibitor, Exerts Cytotoxicity and Histone Alterations via Caspase-8 and FADD in Leukemia Cells. Int J Cell Biol *2010*, 207420.

Rizzatti, E.G., Falcao, R.P., Panepucci, R.A., Proto-Siqueira, R., Anselmo-Lima, W.T., Okamoto, O.K., and Zago, M.A. (2005). Gene expression profiling of mantle cell lymphoma cells reveals aberrant expression of genes from the PI3K-AKT, WNT and TGFbeta signalling pathways. Br J Haematol *130*, 516-526.

Robak, T. (2011). New nucleoside analogs for patients with hematological malignancies. Expert Opin Investig Drugs *20*, 343-359.

Ropero, S., and Esteller, M. (2007). The role of histone deacetylases (HDACs) in human cancer. Mol Oncol *1*, 19-25.

Rosenwald, I.B., Lazaris-Karatzas, A., Sonenberg, N., and Schmidt, E.V. (1993). Elevated levels of cyclin D1 protein in response to increased expression of eukaryotic initiation factor 4E. Mol Cell Biol *13*, 7358-7363.

Rothbart, S.B., and Strahl, B.D. (2014). Interpreting the language of histone and DNA modifications. Biochim Biophys Acta *1839*, 627-643.

Rowley, J.D. (1973). Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. Nature *243*, 290-293.

Rowley, J.D. (2001). Chromosome translocations: dangerous liaisons revisited. Nat Rev Cancer *1*, 245-250.

Rui, L., Emre, N.C., Kruhlak, M.J., Chung, H.J., Steidl, C., Slack, G., Wright, G.W., Lenz, G., Ngo, V.N., Shaffer, A.L.*, et al.* (2010). Cooperative epigenetic modulation by cancer amplicon genes. Cancer Cell *18*, 590-605.

Ruthenburg, A.J., Li, H., Patel, D.J., and Allis, C.D. (2007). Multivalent engagement of chromatin modifications by linked binding modules. Nat Rev Mol Cell Biol *8*, 983-994.

Sado, T., Fenner, M.H., Tan, S.S., Tam, P., Shioda, T., and Li, E. (2000). X inactivation in the mouse embryo deficient for Dnmt1: distinct effect of hypomethylation on imprinted and random X inactivation. Dev Biol *225*, 294-303.

Saito, A., Yamashita, T., Mariko, Y., Nosaka, Y., Tsuchiya, K., Ando, T., Suzuki, T., Tsuruo, T., and Nakanishi, O. (1999). A synthetic inhibitor of histone deacetylase, MS-27-275, with marked in vivo antitumor activity against human tumors. Proc Natl Acad Sci U S A *96*, 4592-4597.

Scacheri, P.C., Crawford, G.E., and Davis, S. (2006a). Statistics for ChIP-chip and DNase hypersensitivity experiments on NimbleGen arrays. Methods Enzymol *411*, 270-282.

Scacheri, P.C., Davis, S., Odom, D.T., Crawford, G.E., Perkins, S., Halawi, M.J., Agarwal, S.K., Marx, S.J., Spiegel, A.M., Meltzer, P.S.*, et al.* (2006b). Genome-wide analysis of menin binding provides insights into MEN1 tumorigenesis. PLoS Genet *2*, e51.

Scandura, J.M., Roboz, G.J., Moh, M., Morawa, E., Brenet, F., Bose, J.R., Villegas, L., Gergis, U.S., Mayer, S.A., Ippoliti, C.M.*, et al.* (2011). Phase 1 study of epigenetic priming with decitabine prior to standard induction chemotherapy for patients with AML. Blood *118*, 1472-1480.

Scarpa, E.S., Fabrizio, G., and Di Girolamo, M. (2013). A role of intracellular mono-ADP-ribosylation in cancer biology. FEBS J *280*, 3551-3562.

Schlesinger, Y., Straussman, R., Keshet, I., Farkash, S., Hecht, M., Zimmerman, J., Eden, E., Yakhini, Z., Ben-Shushan, E., Reubinoff, B.E.*, et al.* (2007). Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. Nat Genet *39*, 232-236.

Schmidt, C., and Dreyling, M. (2008). Therapy of mantle cell lymphoma: current standards and future strategies. Hematol Oncol Clin North Am *22*, 953-963, ix.

Sebat, J., Lakshmi, B., Malhotra, D., Troge, J., Lese-Martin, C., Walsh, T., Yamrom, B., Yoon, S., Krasnitz, A., Kendall, J.*, et al.* (2007). Strong association of de novo copy number mutations with autism. Science *316*, 445-449.

Seligson, D.B., Horvath, S., Shi, T., Yu, H., Tze, S., Grunstein, M., and Kurdistani, S.K. (2005). Global histone modification patterns predict risk of prostate cancer recurrence. Nature *435*, 1262-1266.

Seo, S.Y. (2012). Multi-targeted hybrids based on HDAC inhibitors for anti-cancer drug discovery. Arch Pharm Res *35*, 197-200.

Seto, E., and Yoshida, M. (2014). Erasers of histone acetylation: the histone deacetylase enzymes. Cold Spring Harb Perspect Biol *6*, a018713.

Sewing, A., Burger, C., Brusselbach, S., Schalk, C., Lucibello, F.C., and Muller, R. (1993). Human cyclin D1 encodes a labile nuclear protein whose synthesis is directly induced by growth factors and suppressed by cyclic AMP. J Cell Sci *104 (Pt 2)*, 545-555.

Shaffer, A.L., Rosenwald, A., and Staudt, L.M. (2002). Lymphoid malignancies: the dark side of B-cell differentiation. Nat Rev Immunol *2*, 920-932.

Shahbazian, M.D., and Grunstein, M. (2007). Functions of site-specific histone acetylation and deacetylation. Annu Rev Biochem *76*, 75-100.

Shaknovich, R., and Melnick, A. (2011). Epigenetics and B-cell lymphoma. Curr Opin Hematol *18*, 293-299.

Shankland, K.R., Armitage, J.O., and Hancock, B.W. (2012). Non-Hodgkin lymphoma. Lancet *380*, 848-857.

Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J.R., Cole, P.A., and Casero, R.A. (2004a). Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell *119*, 941-953.

Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J.R., Cole, P.A., Casero, R.A., and Shi, Y. (2004b). Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell *119*, 941-953.

Shilatifard, A. (2006). Chromatin modifications by methylation and ubiquitination: implications in the regulation of gene expression. Annu Rev Biochem *75*, 243-269.

Simon, J.A., and Lange, C.A. (2008). Roles of the EZH2 histone methyltransferase in cancer epigenetics. Mutat Res *647*, 21-29.

Siu, L.L., Pili, R., Duran, I., Messersmith, W.A., Chen, E.X., Sullivan, R., MacLean, M., King, S., Brown, S., Reid, G.K.*, et al.* (2008). Phase I study of MGCD0103 given as a three-times-per-week oral dose in patients with advanced solid tumors. J Clin Oncol *26*, 1940-1947.

Skarbnik, A.P., and Smith, M.R. (2012). Radioimmunotherapy in mantle cell lymphoma. Best Pract Res Clin Haematol *25*, 201-210.

Smeets, D.F., Moog, U., Weemaes, C.M., Vaes-Peeters, G., Merkx, G.F., Niehof, J.P., and Hamers, G. (1994). ICF syndrome: a new case and review of the literature. Hum Genet *94*, 240-246.

Smith, J., Tho, L.M., Xu, N., and Gillespie, D.A. (2010). The ATM-Chk2 and ATR-Chk1 pathways in DNA damage signaling and cancer. Adv Cancer Res *108*, 73-112.

Sneeringer, C.J., Scott, M.P., Kuntz, K.W., Knutson, S.K., Pollock, R.M., Richon, V.M., and Copeland, R.A. (2010). Coordinated activities of wild-type plus mutant EZH2 drive tumor-associated hypertrimethylation of lysine 27 on histone H3 (H3K27) in human B-cell lymphomas. Proc Natl Acad Sci U S A *107*, 20980-20985.

Solovei, I., Walter, J., Cremer, M., Habermann, F.A., Schermelleh, L., and Cremer, T. (2002). FISH: A Practical Approach. In FISH: A Practical Approach, J. Squire, B. Beatty, and S. Mai, eds. (Oxford, Oxford Univ. Press).

Song, J., Noh, J.H., Lee, J.H., Eun, J.W., Ahn, Y.M., Kim, S.Y., Lee, S.H., Park, W.S., Yoo, N.J., Lee, J.Y.*, et al.* (2005). Increased expression of histone deacetylase 2 is found in human gastric cancer. APMIS *113*, 264-268.

Soppe, W.J., Jasencakova, Z., Houben, A., Kakutani, T., Meister, A., Huang, M.S., Jacobsen, S.E., Schubert, I., and Fransz, P.F. (2002). DNA methylation controls histone H3 lysine 9 methylation and heterochromatin assembly in Arabidopsis. EMBO J *21*, 6549-6559.

Steele, N., Finn, P., Brown, R., and Plumb, J.A. (2009). Combined inhibition of DNA methylation and histone acetylation enhances gene re-expression and drug sensitivity in vivo. Br J Cancer *100*, 758-763.

Stresemann, C., Brueckner, B., Musch, T., Stopper, H., and Lyko, F. (2006). Functional diversity of DNA methyltransferase inhibitors in human cancer cell lines. Cancer Res *66*, 2794-2800.

Su, I.H., Basavaraj, A., Krutchinsky, A.N., Hobert, O., Ullrich, A., Chait, B.T., and Tarakhovsky, A. (2003). Ezh2 controls B cell development through histone H3 methylation and Igh rearrangement. Nat Immunol *4*, 124-131.

Sun, H.B., Shen, J., and Yokota, H. (2000). Size-dependent positioning of human chromosomes in interphase nuclei. Biophys J *79*, 184-190.

Sutrala, S.R., Norton, N., Williams, N.M., and Buckland, P.R. (2008). Gene copy number variation in schizophrenia. Am J Med Genet B Neuropsychiatr Genet *147B*, 606-611.

Suzuki, K., Shijuuku, T., Fukamachi, T., Zaunders, J., Guillemin, G., Cooper, D., and Kelleher, A. (2005). Prolonged transcriptional silencing and CpG methylation induced by siRNAs targeted to the HIV-1 promoter region. J RNAi Gene Silencing *1*, 66-78.

Taft, R.J., Pheasant, M., and Mattick, J.S. (2007). The relationship between non-protein-coding DNA and eukaryotic complexity. Bioessays *29*, 288-299.

Takizawa, T., Gudla, P.R., Guo, L., Lockett, S., and Misteli, T. (2008). Allele-specific nuclear positioning of the monoallelically expressed astrocyte marker GFAP. Genes Dev *22*, 489-498.

Talbert, P.B., and Henikoff, S. (2006). Spreading of silent chromatin: inaction at a distance. Nat Rev Genet *7*, 793-803.

Tamaru, H., and Selker, E.U. (2001). A histone H3 methyltransferase controls DNA methylation in Neurospora crassa. Nature *414*, 277-283.

Tan, J., Cang, S., Ma, Y., Petrillo, R.L., and Liu, D. (2010). Novel histone deacetylase inhibitors in clinical trials as anti-cancer agents. J Hematol Oncol *3*, 5.

Tanabe, H., Muller, S., Neusser, M., von Hase, J., Calcagno, E., Cremer, M., Solovei, I., Cremer, C., and Cremer, T. (2002). Evolutionary conservation of chromosome territory arrangements in cell nuclei from higher primates. Proc Natl Acad Sci U S A *99*, 4424-4429.

Taub, R., Kirsch, I., Morton, C., Lenoir, G., Swan, D., Tronick, S., Aaronson, S., and Leder, P. (1982). Translocation of the c-myc gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. Proc Natl Acad Sci U S A *79*, 7837-7841.

Taylor, K.H., Briley, A., Wang, Z., Cheng, J., Shi, H., and Caldwell, C.W. (2013a). Aberrant Epigenetic Gene Regulation in Lymphoid Malignancies. Seminars in Hematology *50*, 38-47.

Taylor, K.H., Briley, A., Wang, Z., Cheng, J., Shi, H., and Caldwell, C.W. (2013b). Aberrant epigenetic gene regulation in lymphoid malignancies. Semin Hematol *50*, 38-47.

Tessarz, P., and Kouzarides, T. (2014). Histone core modifications regulating nucleosome structure and dynamics. Nat Rev Mol Cell Biol *15*, 703-708.

Thieblemont, C., Rolland, D., Baseggio, L., Felman, P., Gazzo, S., Callet-Bauchu, E., Traverse-Glehen, A., Houlgatte, R., Fu, K., Weisenburger, D.*, et al.* (2008). Comprehensive analysis of GST-pi expression in B-cell lymphomas: Correlation with histological subtypes and survival. Leuk Lymphoma *49*, 1403-1406.

Thurn, K.T., Thomas, S., Moore, A., and Munster, P.N. (2011). Rational therapeutic combinations with histone deacetylase inhibitors for the treatment of cancer. Future Oncol *7*, 263-283.

Torchia, J., Glass, C., and Rosenfeld, M.G. (1998). Co-activators and co-repressors in the integration of transcriptional responses. Curr Opin Cell Biol *10*, 373-383.

Troussard, X., Avet-Loiseau, H., Macro, M., Mellerin, M.P., Malet, M., Roussel, M., and Sola, B. (2000). Cyclin D1 expression in patients with multiple myeloma. Hematol J *1*, 181-185.

Tsukada, Y., Fang, J., Erdjument-Bromage, H., Warren, M.E., Borchers, C.H., Tempst, P., and Zhang, Y. (2006). Histone demethylation by a family of JmjC domain-containing proteins. Nature *439*, 811-816.

Vakoc, C.R., Sachdeva, M.M., Wang, H., and Blobel, G.A. (2006). Profile of histone lysine methylation across transcribed mammalian chromatin. Mol Cell Biol *26*, 9185-9195.

Valk-Lingbeek, M.E., Bruggeman, S.W., and van Lohuizen, M. (2004). Stem cells and cancer; the polycomb connection. Cell *118*, 409-418.

van Engeland, M., Derks, S., Smits, K.M., Meijer, G.A., and Herman, J.G. (2011). Colorectal cancer epigenetics: complex simplicity. J Clin Oncol *29*, 1382-1391.

van Haaften, G., Dalgliesh, G.L., Davies, H., Chen, L., Bignell, G., Greenman, C., Edkins, S., Hardy, C., O'Meara, S., Teague, J.*, et al.* (2009). Somatic mutations of the histone H3K27 demethylase gene UTX in human cancer. Nat Genet *41*, 521-523.

Van Lint, C., Emiliani, S., and Verdin, E. (1996). The expression of a small fraction of cellular genes is changed in response to histone hyperacetylation. Gene Expr *5*, 245-253.

Vassetzky, Y.S., Hair, A., and Razin, S.V. (2000). Rearrangement of chromatin domains in cancer and development. J Cell Biochem *S35*, 54-60.

Velichutina, I., Shaknovich, R., Geng, H., Johnson, N.A., Gascoyne, R.D., Melnick, A.M., and Elemento, O. (2010). EZH2-mediated epigenetic silencing in germinal center B cells contributes to proliferation and lymphomagenesis. Blood *116*, 5247-5255.

Vogelstein, B., and Kinzler, K.W. (2004). Cancer genes and the pathways they control. Nat Med *10*, 789-799.

Volik, S., Raphael, B.J., Huang, G., Stratton, M.R., Bignel, G., Murnane, J., Brebner, J.H., Bajsarowicz, K., Paris, P.L., Tao, Q*., et al.* (2006). Decoding the fine-scale structure of a breast cancer genome and transcriptome. Genome Res *16*, 394-404.

Waddington, C. (1942). The epigenotype. Endeavour *1*, 18–20.

Walkinshaw, D.R., and Yang, X.J. (2008). Histone deacetylase inhibitors as novel anticancer therapeutics. Curr Oncol *15*, 237-243.

Wang, H., Zhai, L., Xu, J., Joo, H.Y., Jackson, S., Erdjument-Bromage, H., Tempst, P., Xiong, Y., and Zhang, Y. (2006). Histone H3 and H4 ubiquitylation by the CUL4-DDB-ROC1 ubiquitin ligase facilitates cellular response to DNA damage. Mol Cell *22*, 383-394.

Wang, Z., Zang, C., Cui, K., Schones, D.E., Barski, A., Peng, W., and Zhao, K. (2009). Genome-wide mapping of HATs and HDACs reveals distinct functions in active and inactive genes. Cell *138*, 1019-1031.

Weissmann, F., and Lyko, F. (2003). Cooperative interactions between epigenetic modifications and their function in the regulation of chromosome architecture. Bioessays *25*, 792-797.

West, A.C., and Johnstone, R.W. (2014). New and emerging HDAC inhibitors for cancer treatment. J Clin Invest *124*, 30-39.

Williams, M.E., Swerdlow, S.H., and Meeker, T.C. (1993). Chromosome t(11;14)(q13;q32) breakpoints in centrocytic lymphoma are highly localized at the bcl-1 major translocation cluster. Leukemia *7*, 1437-1440.

Wilson, A.S., Power, B.E., and Molloy, P.L. (2007). DNA hypomethylation and human diseases. Biochim Biophys Acta *1775*, 138-162.

Witt, O., Deubzer, H.E., Lodrini, M., Milde, T., and Oehme, I. (2009a). Targeting histone deacetylases in neuroblastoma. Curr Pharm Des *15*, 436-447.

Witt, O., Deubzer, H.E., Milde, T., and Oehme, I. (2009b). HDAC family: What are the cancer relevant targets? Cancer Lett *277*, 8-21.

Wittschieben, B.O., Otero, G., de Bizemont, T., Fellows, J., Erdjument-Bromage, H., Ohba, R., Li, Y., Allis, C.D., Tempst, P., and Svejstrup, J.Q. (1999). A novel histone acetyltransferase is an integral subunit of elongating RNA polymerase II holoenzyme. Mol Cell *4*, 123-128.

Wlodarska, I., Dierickx, D., Vanhentenrijk, V., Van Roosbroeck, K., Pospisilova, H., Minnei, F., Verhoef, G., Thomas, J., Vandenberghe, P., and De Wolf-Peeters, C. (2008). Translocations targeting CCND2, CCND3, and MYCN do occur in t(11;14)-negative mantle cell lymphomas. Blood *111*, 5683-5690.

Wood, A., and Shilatifard, A. (2004). Posttranslational modifications of histones by methylation. Adv Protein Chem *67*, 201-222.

Wu, S.C., and Zhang, Y. (2010). Active DNA demethylation: many roads lead to Rome. Nat Rev Mol Cell Biol *11*, 607-620.

Xu, W.S., Parmigiani, R.B., and Marks, P.A. (2007). Histone deacetylase inhibitors: molecular mechanisms of action. Oncogene *26*, 5541-5552.

Yang, H., Maddipoti, S., Quesada, A., Bohannan, Z., Cabrero Calvo, M., Colla, S., Wei, Y., Estecio, M., Wierda, W., Bueso-Ramos, C.*, et al.* (2015). Analysis of class I and II histone deacetylase gene expression in human leukemia. Leuk Lymphoma, 1-8.

Yang, X.J. (2004). The diverse superfamily of lysine acetyltransferases and their roles in leukemia and other diseases. Nucleic Acids Res *32*, 959-976.

Ying, Z.T., Zheng, W., Wang, X.P., Xie, Y., Tu, M.F., Lin, N.J., Ping, L.Y., Liu, W.P., Deng, L.J., Zhang, C.*, et al.* (2012). The clinical features, therapeutic responses, and prognosis of the patients with mantle cell lymphoma. Chin J Cancer *31*, 348-353.

Yoshida, M., Kijima, M., Akita, M., and Beppu, T. (1990). Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. J Biol Chem *265*, 17174-17179.

Zain, J.M., and O'Connor, O. (2010). Targeted treatment and new agents in peripheral T-cell lymphoma. Int J Hematol *92*, 33-44.

Zanger, K., Radovick, S., and Wondisford, F.E. (2001). CREB binding protein recruitment to the transcription complex requires growth factor-dependent phosphorylation of its GF box. Mol Cell *7*, 551-558.

Zelent, A., Greaves, M., and Enver, T. (2004). Role of the TEL-AML1 fusion gene in the molecular pathogenesis of childhood acute lymphoblastic leukaemia. Oncogene *23*, 4275-4283.

Zhang, B., Pan, X., Cobb, G.P., and Anderson, T.A. (2007). microRNAs as oncogenes and tumor suppressors. Dev Biol *302*, 1-12.

Zhang, J., Baran, J., Cros, A., Guberman, J.M., Haider, S., Hsu, J., Liang, Y., Rivkin, E., Wang, J., Whitty, B.*, et al.* (2011). International Cancer Genome Consortium Data Portal--a one-stop shop for cancer genomics data. Database (Oxford) *2011*, bar026.

Zhang, P., Guo, Z., Wu, Y., Hu, R., Du, J., He, X., Jiao, X., and Zhu, X. (2015). Histone Deacetylase Inhibitors Inhibit the Proliferation of Gallbladder Carcinoma Cells by Suppressing AKT/mTOR Signaling. PLoS One *10*, e0136193.

Zhang, W., Bone, J.R., Edmondson, D.G., Turner, B.M., and Roth, S.Y. (1998). Essential and redundant functions of histone acetylation revealed by mutation of target lysines and loss of the Gcn5p acetyltransferase. EMBO J *17*, 3155-3167.

Zhou, C., Qiu, L., Sun, Y., Healey, S., Wanebo, H., Kouttab, N., Di, W., Yan, B., and Wan, Y. (2006). Inhibition of EGFR/PI3K/AKT cell survival pathway promotes TSA's effect on cell death and migration in human ovarian cancer cells. Int J Oncol *29*, 269-278.

Zhou, H., Hu, H., and Lai, M. (2010). Non-coding RNAs and their epigenetic regulatory mechanisms. Biol Cell *102*, 645-655.

Zhu, B., Zheng, Y., Pham, A.D., Mandal, S.S., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (2005). Monoubiquitination of human histone H2B: the factors involved and their roles in HOX gene regulation. Mol Cell *20*, 601-611.

Zilberman, D., Cao, X., and Jacobsen, S.E. (2003). ARGONAUTE4 control of locus-specific siRNA accumulation and DNA and histone methylation. Science *299*, 716-719.

Zucca, E., Roggero, E., Pinotti, G., Pedrinis, E., Cappella, C., Venco, A., and Cavalli, F. (1995). Patterns of survival in mantle cell lymphoma. Ann Oncol *6*, 257-262.

Titre : Organisation nucleaire et regulation transcriptionnelle dans les lymphomes

Mots clés : transcription, cancer, lymphome, HDACi, epigénétique, translocation

Résumé : Le lymphome des cellules du manteau (LCM) est un lymphome d'une rare agressivité causée par la translocation chromosomique t(11;14)(q13;q32) qui activet le proto-oncogène cycline D1 (CCND1). Nos et d'autres découvertes récents suggère un mécanisme epigénétique de régulation des gènes dans les LCM plutôt que simplement un simple effet enhancer-promoteur.
Plusieurs nouveaux traitements contre le LCM ont été proposés, y compris les inhibiteurs d'histone deacetylase (HDACis) qui impliquent des mécanismes epigénétique. Dans LMC, les HDACis se sont révélés antiprolifératifs et ils diminuent le niveau de la cycline D1. Jusqu'à présent, les mécanismes d'action des HDACis reste obscurs. Une étude d'état epigénétique sur les loci 11q13 et 14q32 devrait fortement améliorer notre connaissance de ces processus.
L'objectif de ce travail est d'étudier la structure de la chromatine dans le locus réarrangé (11;14) dans des cellules LMC par rapport au locus 11q13 et 14q32 dans les lymphocytes humains normaux. Nous avons ensuite étudié l'effet de différentes HDACis sur le locus réarrangé (11;14) à plusieurs niveaux: l'acétylation / la méthylation des histones ainsi que conformation de chromatine et l'expression des gènes.
Nous avons montré que t(11;14) conduit à la surexpression de *CCND1* avec un groupe de gènes couvrant plus de 15 Mb autour du point de translocation. Les mêmes gènes, sensibles à la dérégulation par la translocation, réagissent au traitement HDACi en augmentant leur expression. Nos résultats indiquent que HDACi stimule la désagrégation de l'hétérochromatine sur l'ensemble du génome, mais les promoteurs de gènes restent à l'abri de ces effets.

Title : Nuclear organization and transcriptional regulation in lymphomas

Keywords : transcription, cancer, lymphoma, HDACi, epigenetics, translocation

Abstract : Mantle cell lymphoma (MCL) is a rare aggressive lymphoma caused by the chromosome translocation between 11 and 14 chromosomes, which lead to activation of the proto-oncogene cyclin D1 (*CCND1*). Our and others recent discoveries suggest epigenetic mechanisms of *CCND1* up-regulation.

Several new treatments are proposed for MCL, including histone deacetylase inhibitors (HDACis) with epigenetic mechanism of action. In MCL cell lines, HDACis were shown to have antiproliferative effects and to decrease cyclin D1 protein levels in the cells, however, till now underlying mechanisms remain obscure. Therefore, a study of epigenetic state in 11q13 and 14q32 loci should significantly advance our knowledge about the mechanisms of cyclin D1 upregulation in MCL.
The purpose of the present work was to study chromatin structure in the rearranged (11;14)(q13;q32) locus in MCL cells as compared to the 11q13 and 14q32 loci in normal human lymphocytes. Furthermore, we studied the effect of different HDACis on the rearranged (11;14) locus at several levels: histone modifications, chromatin conformation and gene expression.
We have shown that t(11:14)(q13;q32) translocation leads to overexpression of *CCND1* along with a group of genes spanning over 15 Mb around the translocation point. The genes, sensitive to deregulation by t(11;14) translocation, react to the HDACi treatment by increasing their expression. Importantly, while HDACi stimulates genome-wide disaggregation of heterochromatin, gene promoters stay shielded from its effect.

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Synthèse de thèse en français

Le lymphome des cellules du manteau (LCM) est un lymphome d'une rare agressivité causée par la translocation chromosomique t(11;14)(q13;q32) juxtaposant le locus de la cycline D1 (*CCND1*) sur le chromosome 11 avec le locus de la chaîne lourde de l'immunoglobuline (IgH) sur le chromosome 14 (Fisher et al., 1995) (**Fig. 1'**). En conséquence, une cycline D1 proto-oncogène devient active alors qu'elle n'est pas exprimée dans les cellules-B normales (Bosch et al., 1994), (Dreyling et al., 1997). L'hypothèse initiale semble indiquer une influence directe du fort enhancer IgH sur le promoteur du gène *CCND1* afin de surexprimer sa transcription (Wang and Boxer, 2005). Quoi qu'il en soit, le locus *CCND1* peut être éloigné jusqu'à 200kb du point de cassure du chromosome. Nous avons montré que le locus 11q13 relocalise depuis la périphérie du noyau jusque au centre actif de transcription et au nucléole (Allinne et al., 2014). Ce phénomène qui mène à l'activation du locus entier, suggère un mécanisme epigénétique de régulation des gènes dans les LCM plutôt que simplement un simple effet enhancer-promoteur.

Plusieurs nouveaux traitements contre le LCM ont été proposés, y compris les inhibiteurs d'histone deacetylase (HDACis) qui impliquent des mécanismes epigénétique (Camara-Clayette et al., 2012). Dans LMC, les HDACis sont décrites comme vaillant des effets antiprolifératifs et diminuant le niveau de la cycline D1 dans la cellule (Heider et al., 2006). Jusqu'à présent, les mécanismes d'action des HDACis reste obscurs. Pour ces raisons, une étude d'état epigénétique sur les loci 11q13 et 14q32 devrait fortement améliorer notre connaissance sur les mécanismes de surexpression de la cycline D1 dans les LMC.

L’objectif de ce travail est d'étudier la structure de la chromatine dans le locus réarrangé (11;14)(q13;q32) dans des cellules LMC par rapport au locus 11q13 et 14q32 dans les lymphocytes humains normaux. Nous avons ensuite étudié l'effet de différentes HDACis sur le locus réarrangé (11;14)(q13;q32) à plusieurs niveaux: l'acétylation / la méthylation des histones de la chromatine ainsi que sa conformation et l'expression des gènes.

Dans la première partie de travail, nous avons utilisé l'analyse ChIP-on-chip pour montrer un fond épigénétique actif dans les deux locus réarrangés (11q13 et 14q32) après la translocation t(11;14) dans LCM (**Fig. 2’**). Analyse des tableaux d'expression génique de base de données GEO et analyse de l'expression génique RT-qPCR ont révélé une surexpression d'un groupe de gènes (y compris *CCND1* et *GSTP1*) dans MCL par rapport aux cellules normales. Ces gènes surexprimés se sont répartis le long de 15 Mb région, et notamment, ils sont situés sur les deux côtés du point de cassure de chromosomes: c'est-à-dire, dans le locus qui est déménagé à der14 et sur le der11 qui n’est pas remplacé.

La méthode de ChIP-on-chip a découvert que ces gènes surexpressées avaient nettement différente signature de modifications de histone H3 Lys9 par rapport au reste du génome. Curieusement, cette signature est différente même dans les cellules de control, c'est-à-dire avant la translocation se produit (**Fig. 3’**).

Dès qu'il est devenu évident que les processus épigénétiques sont impliqués dans la régulation à la hausse de gènes dans LCM, il était intéressant d'évaluer les médicaments épigénétiques qui influencent l'expression des gènes et l'état de la chromatine afin de mieux comprendre les mécanismes sous-jacents de dérégulation dans les lymphomes. Nous avons testé les inhibiteurs de HDAC, une nouvelle classe de médicaments anti-cancéreux, montrés des résultats prometteurs dans les lymphomes remèdes, bien que son mécanisme d'action exact est loin d'être claire.

La conséquence directe de l'application HDACis est une augmentation des niveaux d'acétylation d'histones dans le génome. En effet, immunocoloration avec des anticorps contre marque constitutive de l'hétérochromatine (H3K9me3) a montré l'activation de la chromatine globale après traitement avec Abexinostat: en 24h, montant de l'hétérochromatine considérablement diminué et dans les cellules LCM et dans le contrôle (**Fig. 4'**). Néanmoins, l'analyse ChIP-on-chip n'a pas montré de changements globaux dans l'état d'acétylation des promoteurs de gènes (en LCM et des cellules de contrôle) (**Fig. 5'**). Au lieu de cela, une seule petite proportion de gènes ont révélé une réponse au médicament: les gènes sensibles à une régulation positive par le translocation (11;14) (appelée comme «upregulated»). En outre, l'acétylation dans ce sous-ensemble de gènes se sont comportés de manière différente selon la lignée cellulaire: diminution ou augmentation des niveaux d'Ac dans 1h, puis diminution dans 24h. Notamment, les marques H3K9me2 ainsi réagi intensivement au traitement avec Abexinostat, mais comme acétylation, que dans un petit group de gènes régulés à la hausse. Ainsi, seule petite proportion de gènes a réagi au traitement HDACi, tandis que les autres gènes ont été l'abri de son influence; et les changements observés ont déclenchées par HDACi de manière indirecte.

Ensuite, nous avons testé influence d'Abexinostat sur l'expression de gènes 11q13. RT-qPCR a montré que tous les gènes sensibles à une régulation positive par le translocation (11;14) ont réagi au traitement avec Abexinostat. Principalement, la plupart des gènes avec une expression faible (inférieur de GAPDH) ont augmenté leur expression dans 24h de traitement (**Fig. 6'**). Par exemple, Abexinostat augmenté les niveaux d'expression de *CCND1* dans les cellules de LCM, mais pas dans le contrôle. Le même scénario a été observé dans le lymphome folliculaire, qui a t(11;18) conduisant à la surexpression de Bcl-2. Abexinostat n'a pas changé niveaux d'expression de BCL-2, mais a augmenté et celles

du *CCND1* et *CTSF*, initialement exprimé très fiable dans les cellules de lymphome folliculaire.

Un certain nombre de résultats ont montré la régulation négative de la protéine cycline D1 induite par HDACi (Heider et al., 2006), (Kawamata et al., 2007). Cependant les mécanismes de ce processus ne sont pas compris. Dans la deuxième partie de travail, nous avons étudié l'impact de HDACis sur les niveaux de cycline D1.

En effet, l'analyse FACS a montré que HDACis Abexinostat et TSA ont diminué la quantité de protéine de cycline D1 dans les lignées cellulaires LCM dans 24h de traitement (**Fig. 7'**). Cependant, l'efficacité de la dégradation de la cycline D1 dépendait du type de lignée cellulaire. Considérant que nos résultats ont montré que ces HDACis ne modifient pas l'expression des *CCND1* dans LCM, nous avons cherché des mécanismes possibles dans les autres niveaux.

Nous avons évalué l'état nucléoles utilisant 3D-FISH et avons détecté un niveau significativement plus élevé de cellules avec des nucléoles considérablement réduits à 24h de traitement (LCM et contrôle) (**Fig. 8'**). Cependant, d'analyse des niveaux de 18S + 28S rRNA n'a pas révélé de changements sur le traitement avec Abexinostat (**Fig. 9'**). Ensuite, nous avons vérifié si HDACi pourrait avoir un impact sur la dégradation de la cycline D1. Nous avons inhibé protéasomes dans les cellules LCM et évalué les niveaux de protéine de cycline D1 en utilisant FACS (**Fig. 10'**). Paradoxalement, inhibiteur du protéasome MG132 ainsi conduit à la réduction de la quantité de protéine cycline D1. Ainsi, apparemment Abexinostat n'affecte pas ni translation au niveau du ribosome, ni la dégradation de cycline D1 protéine par les protéasomes.

Dans la troisième partie de travail, nous avons vérifié si Abexinostat aurait les mêmes effets dans les cellules primaires LCM que ceux qui ont été découverts dans des lignées

cellulaires LCM. Pour cela, nous avons testé l'expression des gènes et de l'état de la chromatine dans un échantillon de patient avec LCM et par rapport à des lymphocytes B normaux (NBL) extraites du sang de donneurs (trois échantillons).

Tout d'abord, nous avons remarqué que 11q13 gènes sont surexprimés dans LCM patient par rapport à NBL a la même manière que dans les lignées cellulaires de LCM contre du contrôle (**Fig. 11'**). Ensuite, nous avons appliqué Abexinostat et découvert qu'il a déclenché le même type de changements dans NBL que dans les lignées cellulaires normales: un gène ayant la plus faible expression initiale sensiblement augmenté en 24h de traitement. Cependant, dans les cellules du patient LCM, les gènes ont montré une réponse à Abexinostat tout à fait différente: une partie des gènes réduit son expression déjà dans 1h d'application de médicament, y compris le gène *CCND1*. En dehors de cela, les modifications d'histones à l'intérieur des gènes ont montré aussi d'autres types de réponse au traitement par contraste avec les lignées cellulaires LCM. Pour vérifier les différences observées, plus des échantillons de LCM patients doivent être analysés. Néanmoins, compte tenu du fait que les cellules primaires diffèrent des cellules établies en culture, il est possible de supposer que nous avons assisté à des effets différents d'Abexinostat sur les processus cellulaires due à la déviation de fond biologique dans des lignées cellulaires et des cellules primaires.

De cette façon, nous avons montré que t(11;14)(q13;q32) conduit à la surexpression de *CCND1* avec un groupe de gènes couvrant plus de 15 Mb autour du point de translocation. Les mêmes gènes, sensibles à la dérégulation par la translocation t(11;14), réagissent au traitement HDACi en augmentant leur expression. Nos résultats indiquent que bien que HDACi stimule la désagrégation de l'hétérochromatine sur l'ensemble du génome, les promoteurs de gènes restent à l'abri de ces effets.

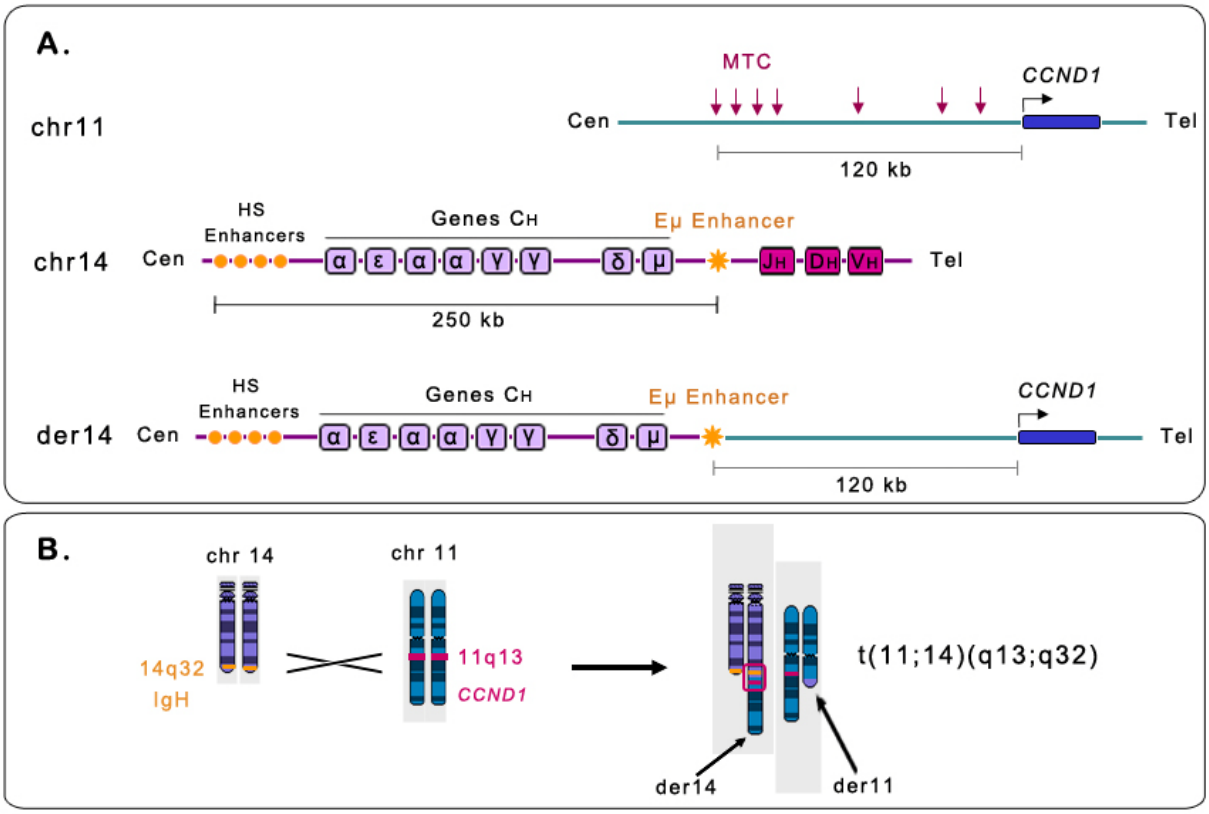
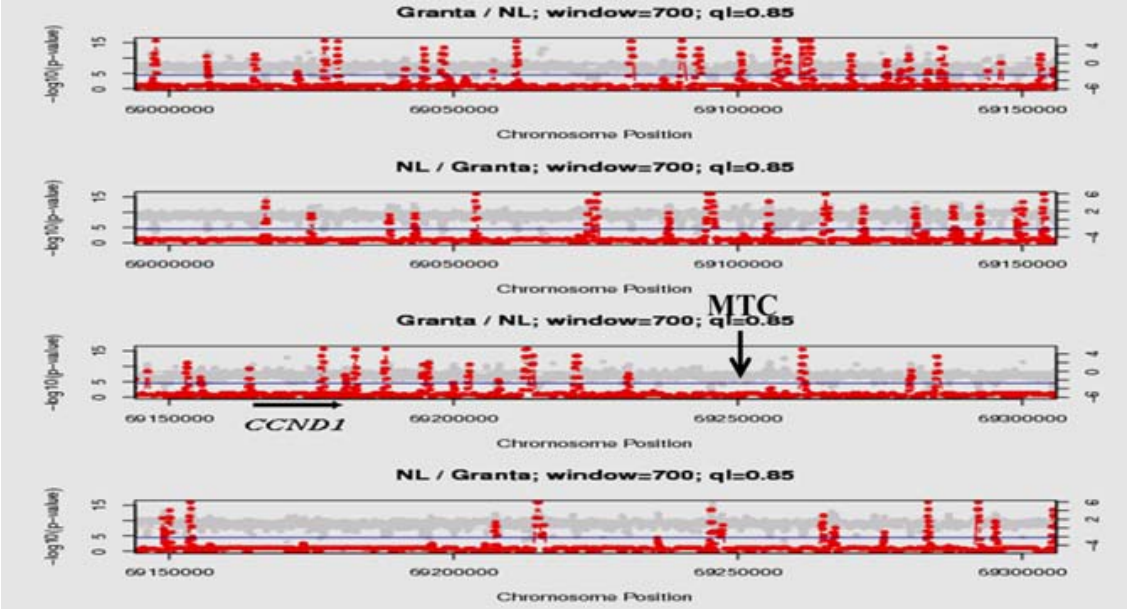


Figure 1'. La translocation t(11;14)(q13;q32) dans le lymphome des cellules du manteau.

A. Structure génomique du locus cycline D1 sur le chromosome 11q13. La plupart des points des ruptures chromosomiques se produisent au sein du région majeure de translocation (major translocation cluster - MTC). Habituellement, la distance entre du point de cassure et le gène *CCND1* est d'environ 120 kb. Le locus normal de la chaîne lourde d'immunoglobuline (IgH) sur le chromosome 14q32 affichage la structure génomique des régions constante (CH), jonction (JH), diversité (DH) et variable (VH). L'activateur Eμ se situe entre les zones constante et jonction. **B**. En raison de la translocation t(11;14)(q13;q32), la cycline D1 est présenté sous le contrôle de l'amplificateur IgH Eμ sur le dérivé du 14 chromosome.

A



B

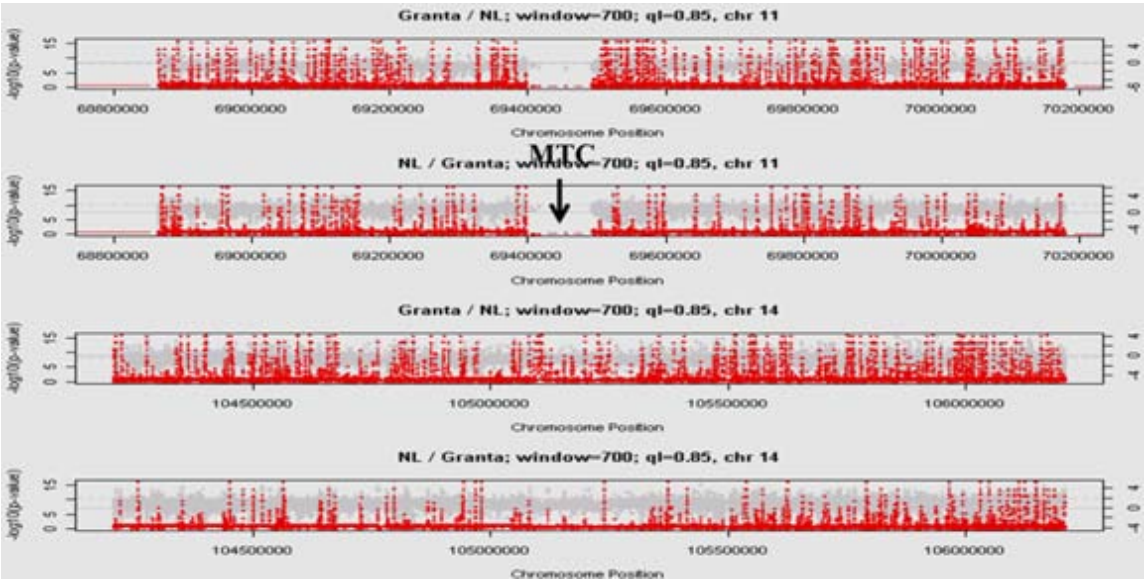


Figure 2'. Distribution des marques H3K9Ac de chromatine dans le locus de translocation.

L'axe X: log2 ratio Granta-519 / NBL (lignes 1er et 3ème) et NBL / Granta-519 (lignes 2ème et 4ème). Les lignes bleues indiquent le niveau de signification plus élevé que les taux de fausses découvertes (FDR); MTC, région majeure de translocation dans MCL. Coordonnées de gènes sont données en correspondance avec l'assemblage HG18. (**A**) Un zoom sur 300Kb segments en 11q13 locus. La flèche horizontale indique le gène *CCND1*. (**B**) Segments analysés entiers: 1.4Mb en 11q13 locus (lignes 1er et 2ème) et 3Mb en 14q32 locus (3ème et 4ème lignes).

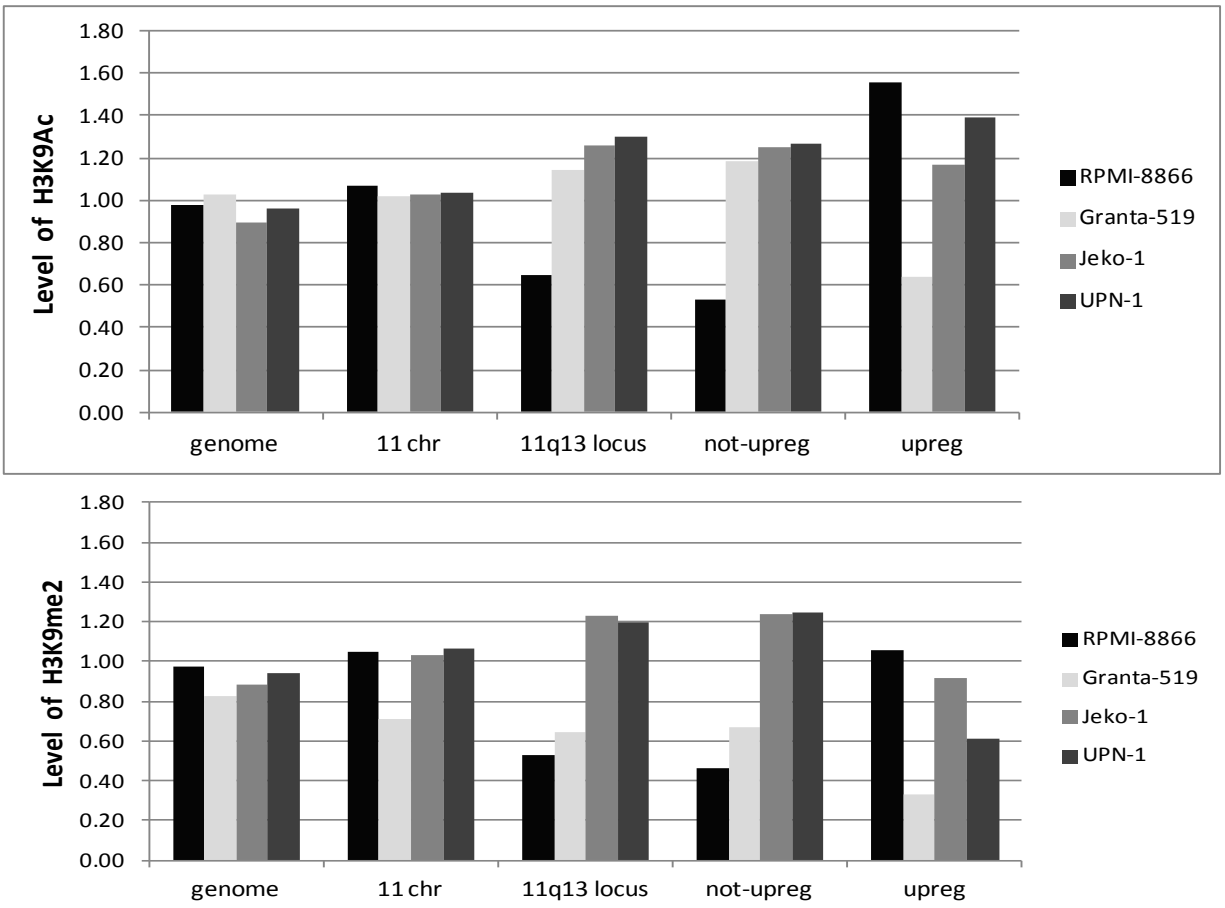


Figure 3'. Niveaux d'acétylation et di-méthylation de H3K9 dans les promoteurs de différents groups de gènes.

Chromatine de la lignée cellulaire de contrôle (RPMI-8866) et de trois lignées de cellules LCM (Granta-519, Jeko-1, UPN-1) a été immunoprécipité avec des anticorps contre H3K9Ac, H3K9me2 et le panH3 comme une référence. Enrichissement en acétylation et méthylation normalisée à panH3 a été estimée à l'aide Agilent humaine Promoteur de puces à ADN. Les pics de H3K9Ac et H3K9me2 significatives statistiquement ont été calculés pour l'ensemble du génome, le chromosome 11, le locus 11q13, pour les gènes qui ne changent pas leur expression après la translocation (non-upreg) et pour les gènes sensibles à une régulation positive après la translocation t(11;14) (upreg). Les données sont présentées en tant que niveau d'acétylation / méthylation (quantité de pics statistiquement significatives de modification d'histone divisé par le nombre de gènes dans la région analysée).

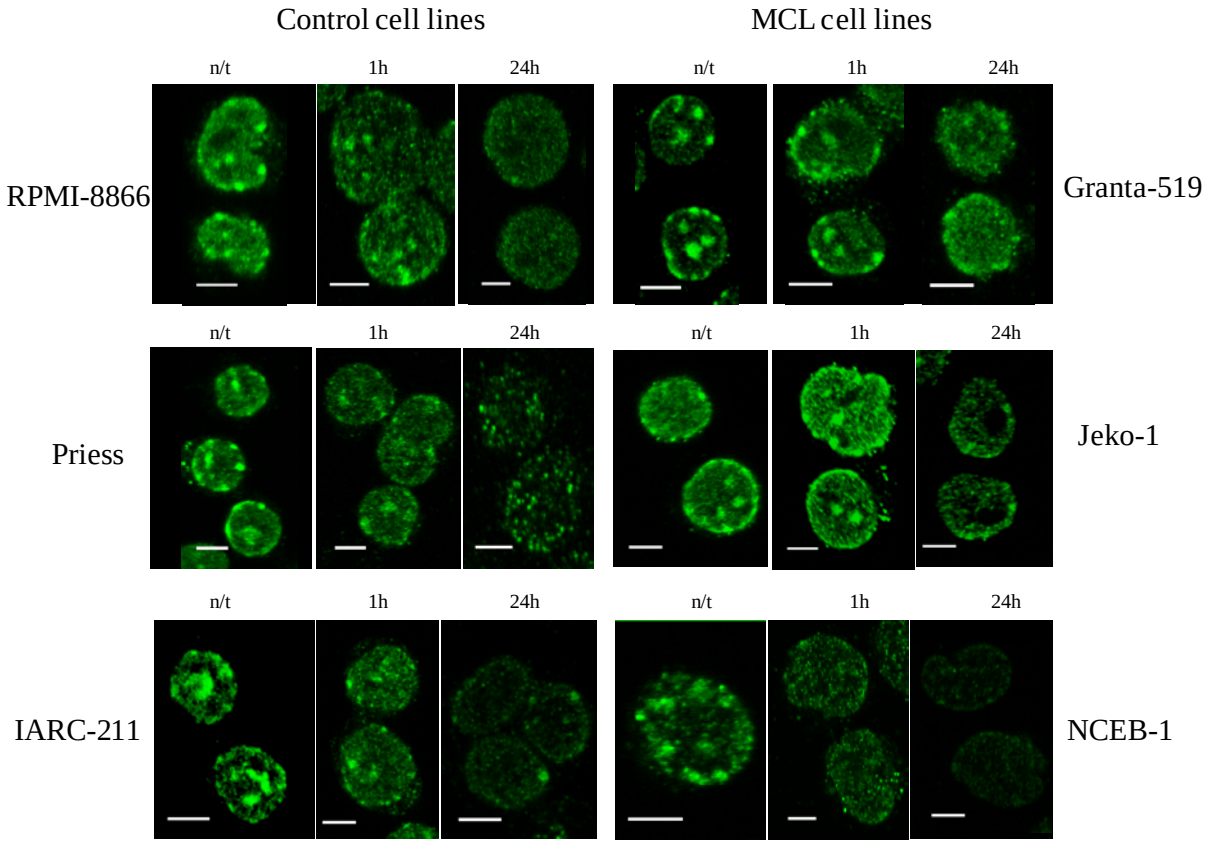


Figure 4'. Les changements dans les niveaux H3K9me3 dans les noyaux de contrôle et de LCM sur le traitement avec Abexinostat.

Les cellules traitée par Abexinostat pendant 1 et 24 heures et les cellules non traitées (n/t) ont été fixées et immunocolorées pour H3K9me3 (vert). La barre d'échelle = 5μM.

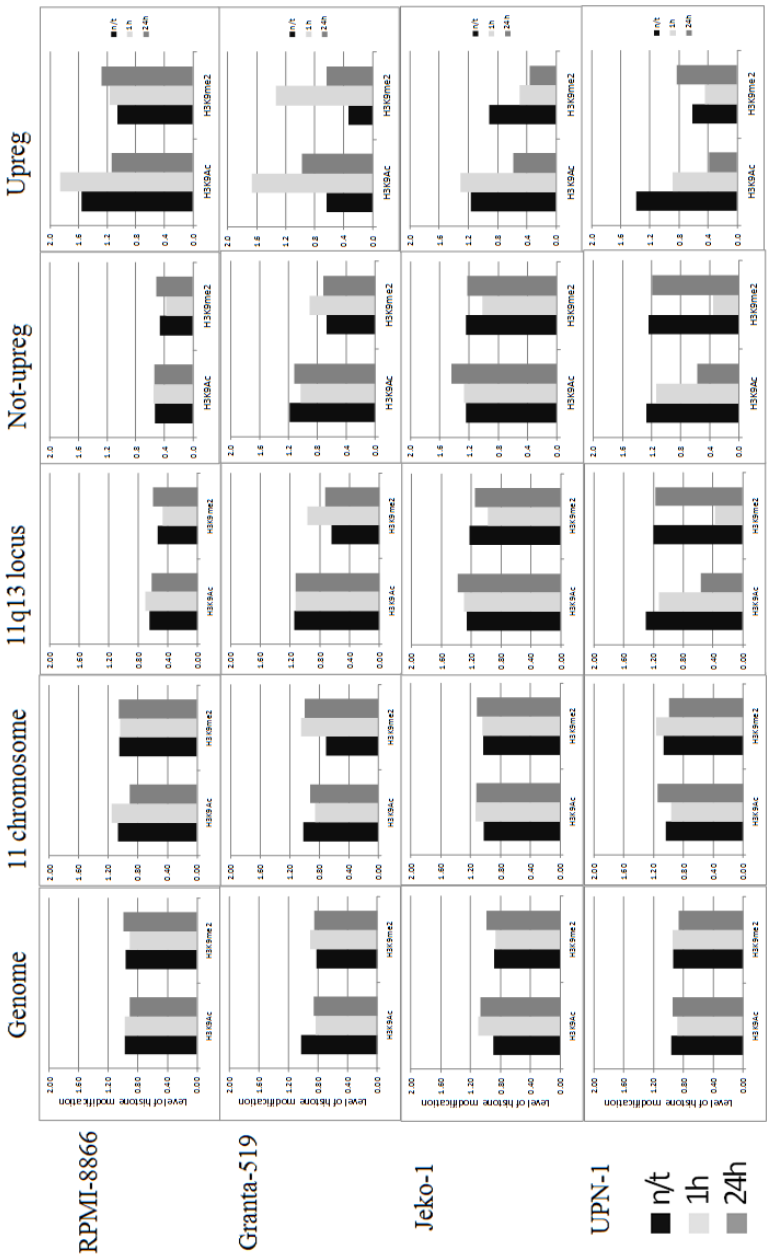


Figure 5'. Changements dans les niveaux d'acétylation et di-méthylation de H3K9 dans les promoteurs de gènes induits par Abexinostat.

Cellules de LCM (Granta-519, Jeko-1, UPN-1) et de contrôle (RPMI-8866) ont été traités avec 100 nM Abexinostat et l'enrichissement H3K9Ac, H3K9me2 normalisée à panH3 a été analysée à des moments définis à l'aide Agilent humaine Promoteur de puces à ADN. Les données sont présentées comme une proposion d'acétylation à méthylation (quantité de pics significatives statistiquement de modification d'histone divisé par le nombre de gènes de la région analysée) dans l'ensemble du génome, le chromosome 11, le locus 11q13, les gènes qui ne modifient pas leur expression après la translocation (non-upreg) et les gènes sensibles à une régulation positive après la translocation t(11;14) (upreg). n/t (noir) - cellules sans traitement; 1h (gris), 24h (gris foncé) - points de temps de abexinostat traitement.

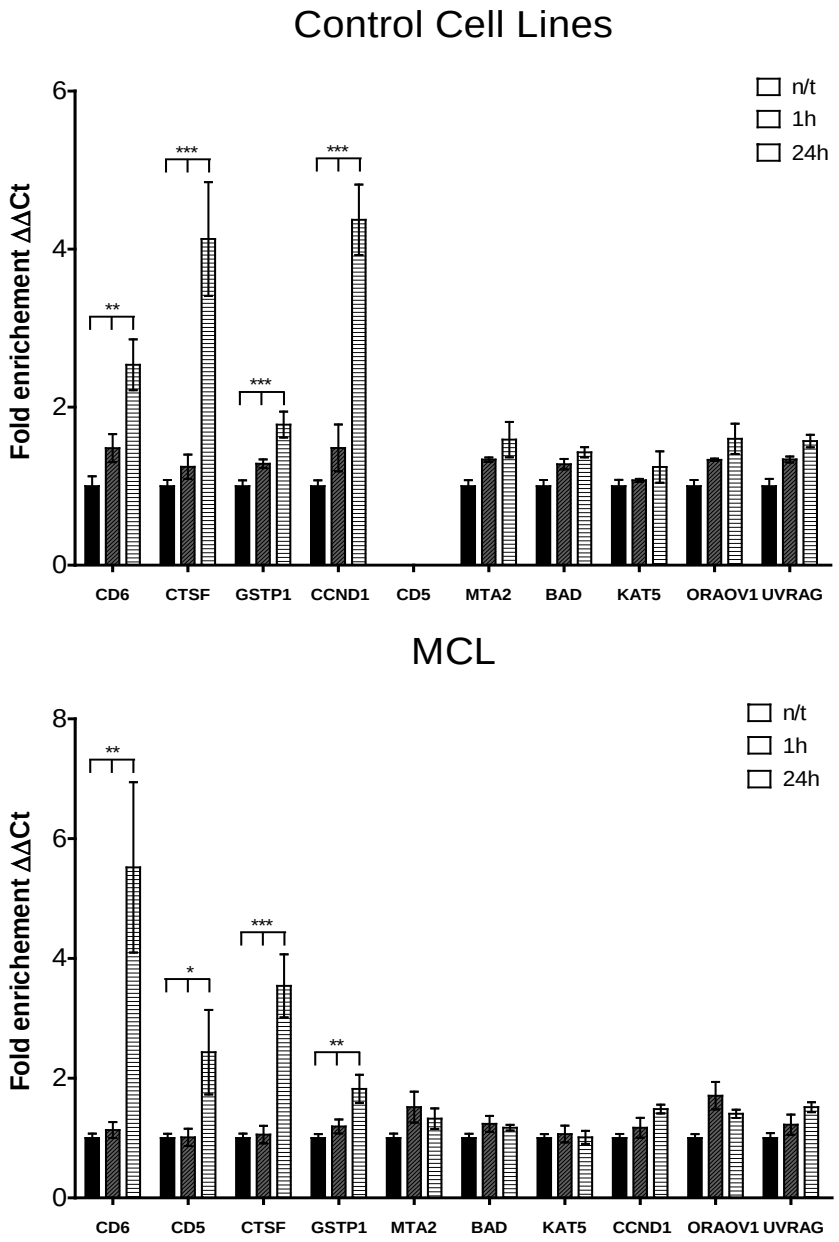


Figure 6'. Effet d'Abexinostat sur les niveaux d'expression de gènes 11q13.

Cinq lignées de cellules de LCM (Granta-519, Jeko-1, UPN-1, Mino et NCEB-1) et cinq de contrôle (RPMI-8866, Priess, Remb 1, le CIRC-211, le CIRC-171) ont été traitées avec 100 nM Abexinostat et les niveaux d'expression de gènes ont été analysés avant le traitement (n/t, noir), à 1h (gris) et 24h (blanc) après le traitement. Le niveau d'expression a été mesuré par RT-qPCR vs. expression de *GAPDH*. Les données représentent la moyenne de cinq MCL et 5 des lignées cellulaires de contrôle. Au moins 3 expériences indépendantes pour la chaque lignées de cellule ont été effectuées. Les valeurs sont présentées sous forme de moyenne ± SEM. * p<0,05; ** p<0,01; *** p<0,001 (1 way ANOVA avec post-test Turkey).

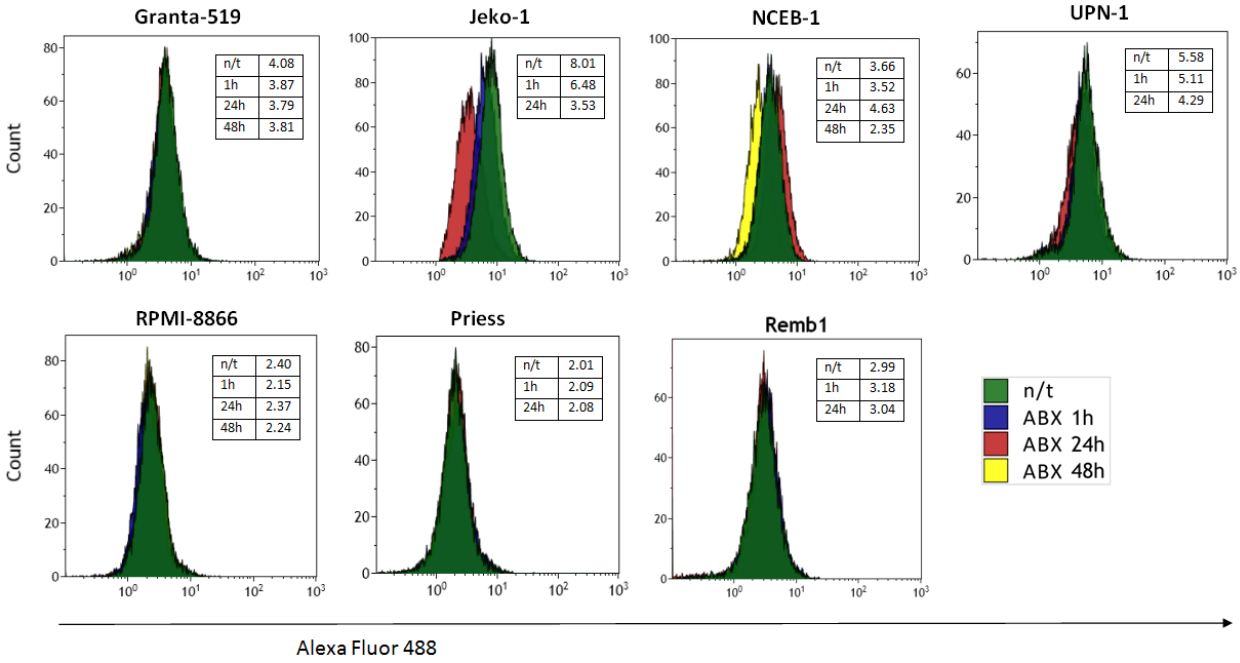
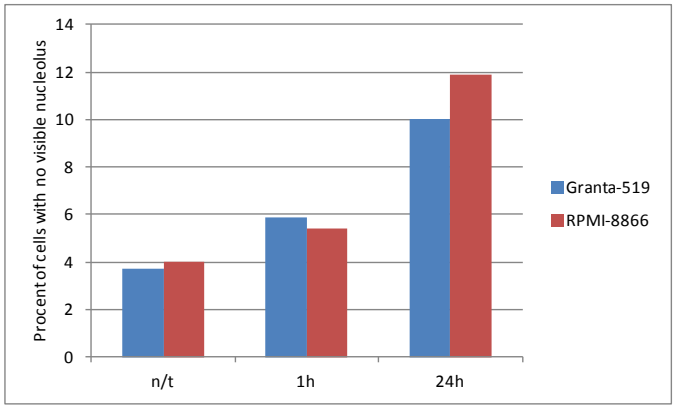
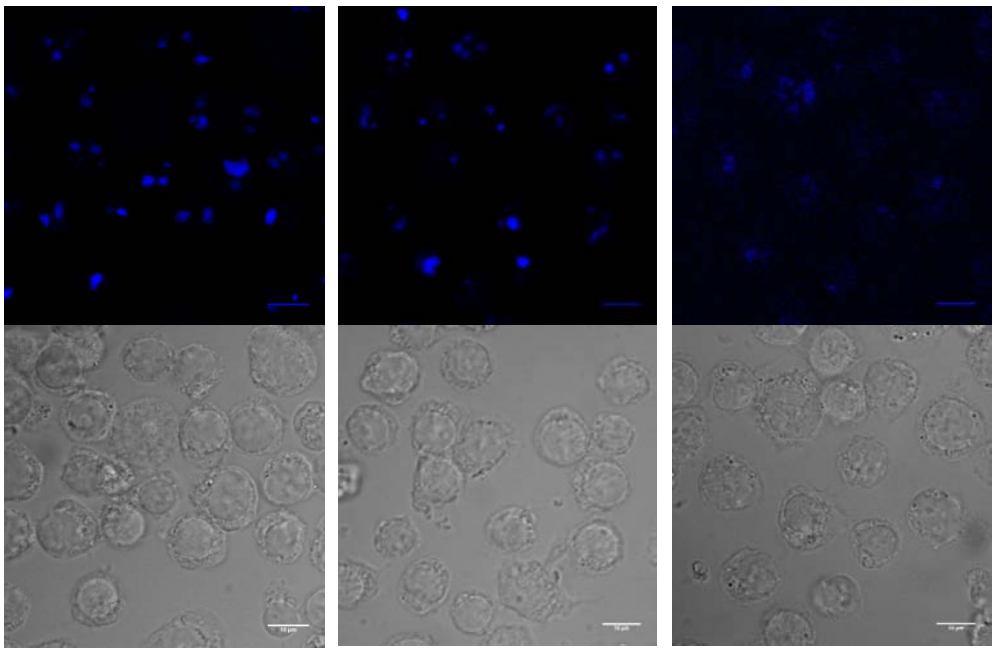


Figure 7'. Histogrammes représentant de cytométrie en flux de réponse de la cycline D1 protéines au traitement par Abexinostat dans les lignées de cellules de LCM et le contrôle.

L'intensité de signal fluorescent de Alexa Fluor 488 est donnée dans les tableaux comme la médiane.



(**A**)



(**B**)

Figure 8'. Le nombre de cellules sans nucléoles visibles a augmenté a 24 heures après de traitement par Abexinostat.

RPMI-8866 et Granta-519 cellules ont été fixées après 1h et 24h du traitement par Abexinostat. Immunocoloration a été faite pour visualiser nucléoles (protéine B23). (**A**) Le graphique montre le niveau de cellules avec des nucléoles réduites à 0, 1 et 24 heures après du traitement par Abexinostat. La courbe en pointillés correspond à la lignée cellulaire Granta-519 et la courbe continue de lignée cellulaire RPMI-8866. (**B**) Une section confocale des cellules Granta-519 colorées avec des anticorps contre un B23 marqueur nucléolaire (bleu). n/t - non traités; 1h et 24h - Abexinostat traité. Barre d'échelle: 5μm.

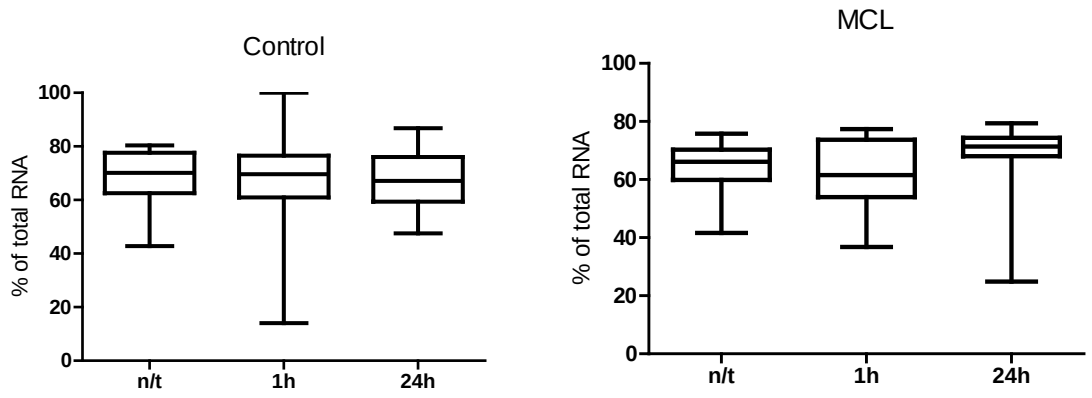


Figure 9'. Dynamique de quantité d'ARNr dans les cellules traitées par Abexinostat.

5 lignées de cellules de LCM et 5 de contrôle ont été traitées avec Abexinostat pendant 1 à 24 heures. L'ARN total a été extrait des cellules traitées et non traitées. Niveau relatif de 18S + 28S ARNr à total ARN a été mesurée à l'aide Bioanalyseur, Agilent Technologies, Inc. La boîte représente les premier et troisième quartiles, la bande à l'intérieur de la boîte est la médiane, les extrémités des moustaches représentent le minimum et le maximum de l'ensemble des données.

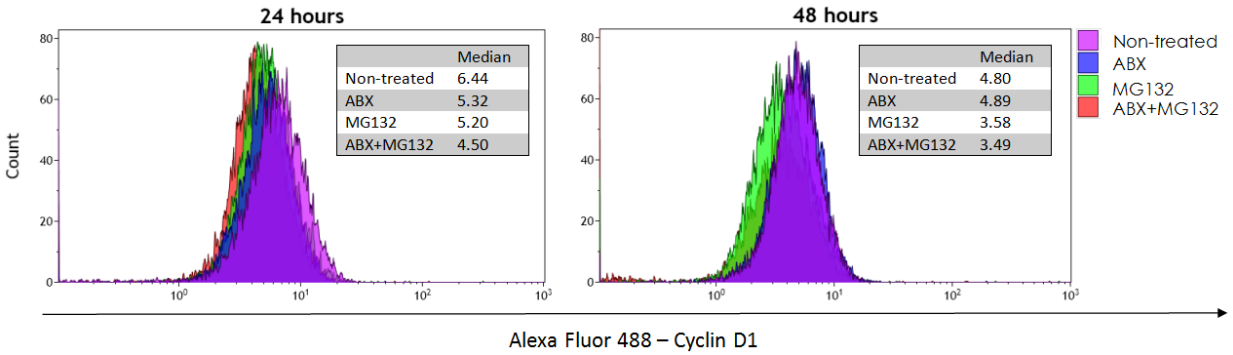
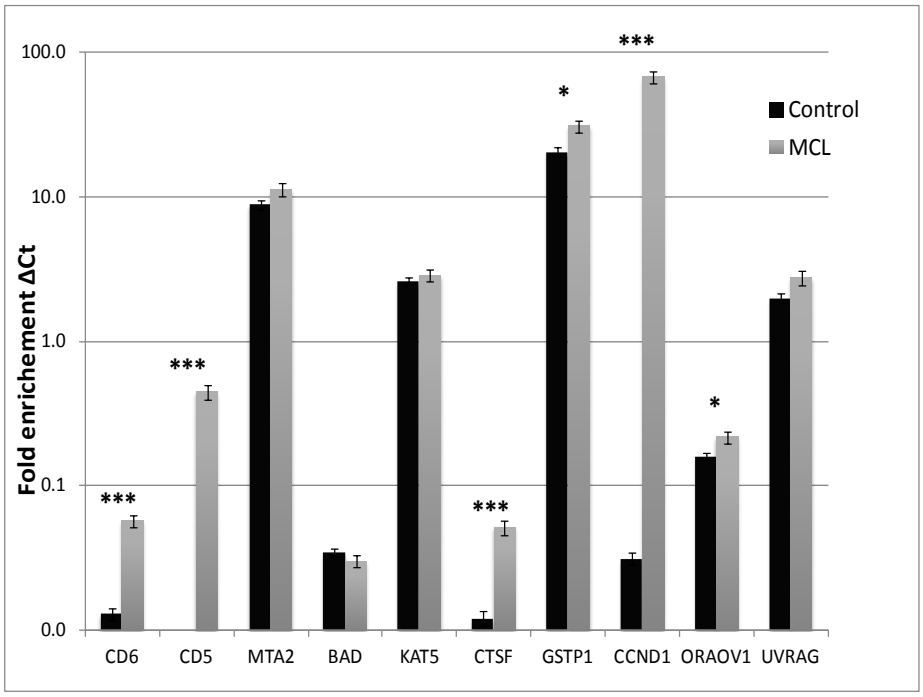
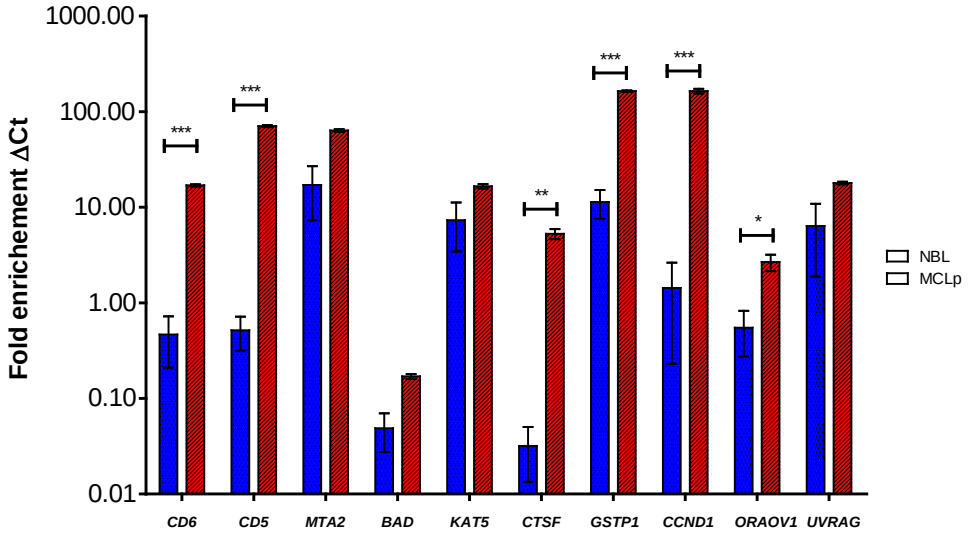


Figure 10'. Effet de l'inhibiteur de protéasome MG-132 sur le niveau de protéine cycline D1 en lignée cellulaire Jeko-1.

Jeko-1 cellules ont été traitées avec MG132 25mM pendant 24h ou 48h. Les niveaux de protéine cycline D1 ont été mesurées à l'aide de FACS en utilisant des anticorps conjugués au FITC contre la cycline D1. Médiane de l'intensité de la cycline D1 fluorescente (Alexa Fluor 488) est représenté dans les tableaux.



(A)



(B)

Figure 11'. Les niveaux d'expression des gènes sélectionnés dans le 11q13 au tour de point de translocation (11;14).

Le graphique indique les niveaux moyenne d'expression des gènes mesurée par RT-qPCR de (**A**) 5 lignées cellulaires de contrôle (noir) et 5 lignées de cellules LCM (gris); et de (**B**) trois personnes sains (NBL, bleu) et les cellules LCM d'un patient (MCLp; rouge). L'abondance de la transcription est mesuré par rapport de *GAPDH*, et présenté sur une échelle logarithmique en base 10. La valeur 1 correspond à l'expression de *GAPDH*. Les données sont présentées sous forme de moyenne ± SEM. * p<0,05; ** p<0,01; *** p<0,001 (test *t* de Student non apparié relative à contrôle).